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(54) Title: IMMUNOGENS FOR STIMULATING MUCOSAL IMMUNITY

(57) Abstract

This patent application relates to immunogens for stimulating mucosal immunity to a pathogen capable of infecting its host through contact with mammalian mucosal membranes. In particular, this invention discloses a number of polypeptides and genetic constructs that include a membrane binding polypeptide operably linked to a peptide from a pathogen. Methods are detailed throughout the claims and the specification for introducing these immunogens into a mammal to stimulate mucosal immune responses.

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IMMUNOGENS FOR STIMULATING MUCOSAL IMMUNITY

FIELD OF THE INVENTION

This application relates to methods for producing mucosal antibody to organisms capable of infecting their host through contact with mammalian mucosal membranes. In particular, this application relates to protein complexes and to gene constructs suitable for producing immunogen capable of inducing mucosal antibody as well as to methods for introducing the immunogen into a mammal to generate a mucosal immune response.

BACKGROUND OF THE INVENTION

The mucosal surfaces of the body are generally accessible to a wide variety of infectious agents capable of causing disease. These surfaces include the gastrointestinal tract, the urogenital tract and the respiratory tract. While the external surfaces of the body are protected by a continuous layer of keratinized squamous epithelium, the mucosal surfaces of the body lack the protective keratinized layer and are more vulnerable to invasion by adventitious organisms. Not surprisingly, these surfaces are the main portal of entry into the body for foreign microorganisms.

The mucosal surfaces of the body have an extensive immune system. Non-encapsulated lymphoid tissue is dispersed throughout the mucosa of the gastrointestinal and genitourinary tracts as either diffuse aggregates of cells or as organized nodules. The diffuse aggregates of lymphoid cells are dispersed throughout the lamina propria while nodules, or Peyer's patches, which include germinal centers of proliferating B cells and peripheral areas of T cell activity, are more prevalent in some regions of the mucosa than in others.

The lymphoid cells along the mucosal surfaces are capable of responding to foreign antigen. The gut epithelium overlying the Peyer's patches allows transport of antigens into the lymphoid tissue and is capable of functioning as antigen presenting cells (Bromander, et al. *Scand. J. Immunol.* 37:452-458, 1993). Secretory IgA (sIgA) can traverse mucosal membranes and is often the first defense that an adventitious agent encounters when contacting the mucosal surface of a mammal.

A vaccine which is effective in preventing diseases which are associated with the invasion of adventitious organisms into the mucosa will preferably stimulate IgG and sIgA. An effective vaccine for limiting mucosal infection will likely demonstrate sIgA activity.

Many sexually transmitted diseases such as chlamydia, gonorrhea, syphilis, chancroid and trichomoniasis are caused by organisms that enter the body through the mucosal membranes. While these diseases are caused by different organisms and replicate in different ways, these sexually transmitted microorganisms, and others, all enter the body primarily via the mucosal barrier. These and most other disease causing organisms carry unique antigenic determinants that are known to stimulate the immune system. In addition, the mucosal surfaces are also the main portal of entry for most viruses. Thus, mucosal surface immunity to viruses including, but not limited to influenza, papillomavirus, HIV, members of the Herpesvirus family, and the like, also integrally associate with mucosal surfaces of the body during the infection stage, replication and as part of virus egress. Attempts to develop vaccines for these organisms have met with little success since parenteral vaccination does not generally produce significant levels of secretory immunity.

The non-viral sexually transmitted diseases can usually be cured if they are diagnosed early, but many of the diseases produce mild early symptoms, if any, and thus go untreated until more advanced symptoms occur.

Chlamydia is a useful example of a sexually transmitted disease that infects its host through mucosal membranes, primarily of the genitourinary system. *Chlamydia trachomatis* is the leading sexually transmitted organism in the United States, afflicting an estimated four million people a year (Division of STD/HIV Prevention, 1992 Annual Report, CDC, Atlanta, 1993). Chlamydia is acquired chiefly through vaginal or anal intercourse, although it can also be transmitted through oral sex. *C. trachomatis* infection of the genital tract can cause salpingitis in women that can result in tubal blockage and infertility. It is estimated that in the United States 200,000 women per year become infertile as a result of chlamydial salpingitis. Moreover, infected individuals are at an increased risk of acquiring HIV if exposed to the virus (Wasserhiet. "Epidemiological Synergy: Interrelationships Between Human Immunodeficiency Virus Infection and Other Sexually Transmitted Diseases," *Sexually Transmitted Diseases*, 19: 61-77, 1992). Improved methods for controlling the pathological manifestations of this disease are seriously needed.

Chlamydia trachomatis isolates occur as 15 distinct serovars that are divided into three subgroups. The major outer membrane protein (MOMP) of Chlamydia confers serovar and serogroup-specificities. Protective immunity to Chlamydia is directed to the major outer membrane protein.

Comparative analysis of the major outer membrane protein of Chlamydia indicates that the MOMP genes are conserved and have four variable region domains that are unique to each serovar. These domains elicit the formation of serovar, subspecies, and serogroup or species-specific antibodies. Variable domain IV (VDIV) is the largest of the MOMP variable domains and is located near the C-terminus of the protein. VDIV contains subspecies, serogroup antigenic determinants and a conserved species-specific antigenic determinant. All of the variable domains of MOMP are external epitopes as demonstrated by their sensitivity to trypsin and by their accessibility to antibody binding. This invention contemplates combining determinants from MOMP or other protein from pathogens, including but not limited to HIV, hepatitis and enterotoxigenic *E. coli* capable of infecting a mammalian host through contact with mucosal membrane with a mucosal binding polypeptide.

Several agents have proved effective as carriers and as adjuvants for stimulating mucosal immunity (for a review of these agents see Bienenstock, J. "The Nature of Immunity at Mucosal Surfaces - A Brief Review." In: Bacterial Infections of Respiratory and Gastrointestinal Mucosae. Eds. Doncachie, W. et al. IRL Press, 1988. pp. 9-18.). Accessible epitopes from various infectious agents have been engineered onto the surface of attenuated live vectors such as Vaccinia or *Salmonella typhimurium* (see Flexner, et al. "Vaccinia as a live vector carrying cloned foreign genes. In: New Generation Vaccines Eds. Woodrow, G.C., et al. Marcel Dekker, Publ. New York 1990, pp. 189-206 and Curtiss, R. "Attenuated Salmonella strains as live vectors for the expression of foreign antigens." Ibid, pp. 161-188). Non-living carrier systems allow immunologically accessible epitopes to be presented to the immune system as the product of a genetic construct or as a peptide chemically coupled to a carrying agent. Such non-living carrier systems include microparticles, liposomes, solid matrix antibody-antigen complexes, immunostimulating complexes (ISCOMs) and protein carriers including the core antigen of hepatitis B virus, polio virions, cholera toxin and the heat-labile enterotoxin from E. coli.

The carriers themselves may have endogenous adjuvant activity or alternatively, exogenous adjuvant can be used with the carriers. Ox bile has been used as an adjuvant for orally administered immunogens such as killed oral vaccines against dysentery. Other adjuvants that have been tested for use in inducing mucosal immunity include DEAE-4 dextran, lysozyme, polyornithine, sodium dodecyl benzene sulfate, lipid-conjugated substances, streptomycin and vitamin A (see Holmgren, et al. *Vaccine* 11:1170-1184, 1993). In addition, agents such as muramyl dipeptide, acridine and cimetidine have had some positive effect as well (see Bienenstock, et al. *supra*.).

Cholera toxin is capable of generating mucosal immunity and is also a potent adjuvant for augmenting the immunizing effect of orally administered vaccines. Cholera toxin is produced by *Vibrio cholerae* bacteria. The toxin molecule is well characterized and in its native form consists of five binding B subunits assembled as a ring together with a single A subunit. The B subunits bind to GM1 receptors on the cell surface. The A subunit is translocated to the inside of the cell following B subunit binding. Cholera toxin has been used both as an immunogen for the oral mucosa and as a potent adjuvant for inducing secretory IgA (Lycke, N. and Holmgren, J. *Immunology* 59:301-308, 1986) to cholera toxin and to unrelated antigen. Similarly, the heat-labile enterotoxin of *Escherichia coli* also has immunomodulating and adjuvant properties.

Cholera toxin coupled to foreign antigen has been used to stimulate immune responses in the gastrointestinal tract. For example, chemical coupling of the cholera toxin B subunit to streptococcal antigen has evoked antibody responses in the mucosal surfaces of the gut and in salivary glands (Czerkinsky, et al. *Infect. Immun.* 57:1072-1077, 1989). Cholera toxin/Sendai virus conjugate immunization has resulted in the production of Sendai-specific immunoglobulin in the respiratory tract.

While cholera toxin has been used to stimulate the immune response to bacterial and select viral antigens along the gastrointestinal tract, there is no successful strategy available for producing total mucosal immunity or effective immunity along the mucosal surfaces of the urogenital tract. Moreover, even where cholera toxin, or its subunits, was used to stimulate immune responses to foreign antigen, the immune responses have been poor as compared with the immune responses generated to the cholera toxin carrier. For example, the addition of amino acid residues to either the N- or C- terminus of CTB has in some cases interfered with the folding and assembly of the final product (Sandkvist, et al., *J. Bacteriol.* 169:4570-4576, 1987 and Clements J.D. *Infect. Immun.* 58:1159-1166, 1990). When some of these constructs were produced in *V. cholerae* the foreign epitopes were cleaved off, presumably by bacterial proteases (Schödel, et al., *Gene* 99:255-259, 1991). Therefore, there remains a need for improved cholera toxin derived immunogens.

Unlike the prior art, protein complexes and genetic constructs are disclosed in this invention that stimulate mucosal immunity to pathogens which cause sexually transmitted disease. In addition, genetic constructs are disclosed that provide increased stability to foreign epitopes linked to mucosal binding polypeptide. Protein complexes are disclosed that are expressed in whole or in part from *E. coli* or *V. cholerae*.

Thus, the present invention not only discloses strategies for the production of mucosal immune responses to non-viral sexually transmitted pathogens and to a variety of viral pathogens but the invention also discloses strategies for improving the immune response to the foreign antigen using a variety of genetic constructs.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 diagrams the strategy for producing the pMLλ *E. coli* expression vectors of the present invention.

Figure 2 provides preferred examples of expression vectors using the *tac* promoter. Figure 2(a) diagrams a plasmid expressing CTB from the *tac* promoter. Figure 2(b) illustrates the same vector as in Figure 2(a), additionally carrying the *ctx*AB gene fragment used for the construction of CTA2 protein fusions. Figure 2(c) diagrams a plasmid, as disclosed in Figure 2(b), additionally containing the *lacI*^{q} gene to facilitate inducible X-gal expression of *ctx*AB. Figure 2(d) diagrams a plasmid carrying the modified *ctx*B gene for in-frame internal epitope insertion between the unique KpnI and MscI sites.

Figure 3 diagrams plasmids expressing CTB from the leftwards promoter of phage lambda for the generation of CTB fusions (pML-LCTBλ7) or CTA fusions (pPJλ).

Figure 4 illustrates the oligonucleotide sequence, SEQ ID NO:5, used to regenerate the *ctx*A signal peptide and the unique restriction sites used for cloning foreign epitopes into the construct.

Figure 5 provides synthetic oligonucleotide sequences, SEQ ID NO:24 and SEQ ID NO:25, used for the insertion of chlamydial T-cell and B-cell epitopes into the *ctx*A gene. Boxed sequences represent the chlamydial peptides.

Figure 6 provides a diagram of the oligonucleotide sequences, SEQ ID NO:7 and SEQ ID NO:8 which were used to form the linker to join the chlamydial epitopes A8 (SEQ ID NO:6) and VDIV (SEQ ID NO:1). The adjacent SacI and NheI sequences for pML-LCTB*tac* or pML-LCTBλ and pPJVDIV are also diagramed in this Figure.

Figure 7 diagrams the construction of plasmid pCB55-64gp309 encoding an exemplary CTB::hybrid protein. The diagram provides an illustration of the parent plasmid, pML-LCTB*tac*, positioned to accept foreign antigenic sequences. Plasmid pCB55-64gp309 contained the HIV antigenic sequence derived from amino acids 309-318 of gp120. The gp120 nucleic acid sequence positioned between the KpnI and BssHII restriction endonuclease sites is provided in the enclosed box with insert gp120 sequences shown in italics.

SUMMARY OF THE INVENTION

This invention relates to immunogens useful for stimulating mucosal immunity and for protein complexes that are useful in assays to detect the presence of antibody to mucosal binding proteins or to foreign antigen from pathogens capable of infecting a mammalian host through mucosal membranes. In addition, the invention relates to methods for inducing mucosal surface immunity to a pathogen capable of infecting a mammalian host through mucosal membranes.

In one embodiment of this invention a mucosal binding composition is contemplated that comprises a mucosal binding polypeptide linked to at least one antigen from a non-viral pathogen where the pathogen causes a sexually transmitted disease. In a preferred embodiment the mucosal binding polypeptide is the binding subunit of cholera toxin and in another embodiment the mucosal binding polypeptide additionally comprises at least a portion

of the A subunit of cholera toxin. In a preferred embodiment the antigen from the non-viral pathogen is an antigen from *Chlamydia*.

It is contemplated that the chlamydia antigen can be positioned at the amino terminus of the binding subunit of the cholera toxin, at the amino terminus of the portion of the A subunit of cholera toxin or positioned internally within the binding subunit of cholera toxin. It is contemplated that the antigen can be linked to the mucosal binding protein by recombinant or chemical means.

In a preferred embodiment, the antigen comprises a B-cell stimulating antigen from the major outer membrane protein of chlamydia. In a particularly preferred embodiment, the B-cell stimulating antigen is from the VDIV region of the major outer membrane protein of chlamydia. In yet another preferred embodiment, the antigen further comprises a T-helper cell stimulating antigen and preferably, this T-helper cell stimulating antigen is also from the major outer membrane protein of chlamydia. In one embodiment the T-helper cell stimulating antigen is from the major outer membrane protein of chlamydia and in a preferred embodiment is the A8 region from the major outer membrane protein of chlamydia.

In another embodiment of the present invention, a method is disclosed for generating a mucosal immune response against a non-viral sexually transmitted disease, comprising contacting the mucosa of a mammalian host with the mucosal binding composition.

In yet another embodiment of the present invention, recombinant polynucleotides are disclosed which comprise a first region encoding a mucosal binding polypeptide and a second region encoding an antigen of a non-viral pathogen, where the pathogen causes a sexually transmitted disease. In a preferred embodiment the mucosal binding polypeptide is the binding subunit of cholera toxin and the pathogen is chlamydia. In this embodiment, the preferred antigen includes a T-helper cell stimulating antigen and a B-cell stimulating antigen from the outer membrane protein of chlamydia. In a particularly preferred embodiment, the B-cell stimulating antigen is from the VDIV region of the major outer membrane protein of chlamydia.

This invention also relates to methods for vaccinating a mammal against chlamydia infection comprising administering to the mucosa of a mammalian host an effective amount of the binding subunit of cholera toxin linked to both a B-cell epitope and a T-cell epitope of the major outer membrane protein of chlamydia. In a preferred embodiment the administration is vaginal and in other embodiments, the vaccine is delivered rectally or orally.

The invention additionally relates to mucosal binding compositions comprising a mucosal binding polypeptide linked to at least one antigen of a viral pathogen where the pathogen causes a sexually transmitted disease. In a preferred embodiment the mucosal binding polypeptide comprises the binding subunit of cholera toxin and the antigen is a HIV gp120 antigen. In yet another embodiment the antigen is a Hepatitis B virus pre-S(2) antigen and in a further embodiment, the mucosal binding polypeptide is linked to at least one antigen from the ST_1 protein of enterotoxigenic *E. coli*.

The invention additionally contemplates purified recombinant polynucleotides comprising nucleic acid encoding a mucosal binding protein operably linked to a B-cell stimulating antigen where the antigen is a peptide obtained from a pathogen capable of infecting a mammal through the mucosal membranes. In a preferred embodiment, the mucosal

binding protein encodes the binding subunit of cholera toxin and in another embodiment, the nucleic acid further encodes the CTA(2) subunit of cholera toxin. In a preferred embodiment the B-cell stimulating antigen encodes a peptide which includes the amino acid sequence LNPTIAG. In one embodiment, the B-cell stimulating antigen is from HIV gp120.

In yet another preferred embodiment, the nucleic acid encoding the B-cell stimulating antigen is positioned in-frame within the coding region of the nucleic acid encoding the mucosal binding protein. The B-cell stimulating antigen may alternatively include the amino acid sequence LNPTIAG, an antigen from the gp120 protein of HIV, the Hepatitis B virus pre-S(2) protein or the ST_a protein of enterotoxigenic *E. coli*. In these embodiments it is contemplated that the nucleic acid encoding the B-cell stimulating antigen is between 21 and 150 bases in length and more preferably between 21 and 60 bases in length.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to immunogens useful for producing mucosal immune responses to a variety of viral and non-viral pathogens, to methods for preparing these immunogens and to methods for producing mucosal immune responses to pathogens capable of infecting a mammalian host through the mucosal membranes of the host.

The term "immunoaccessible" is used herein to describe antigenic sequences that are accessible to the immune system when introduced in association with a mucosal binding polypeptide.

The term "T-cell stimulating antigen" or "T-cell antigen" is used herein to refer to a peptide, a polypeptide, a protein or a nonproteinaceous molecule including carbohydrate, lipid, nucleic acid or the like, which is capable of stimulating T-helper cell activity in standard T helper cell assays, well known in the art, either alone or in combination with other protein.

The term "B-cell stimulating antigen" or "B-cell antigen" is used herein to refer to a peptide, a polypeptide, a protein or a nonproteinaceous molecule, including carbohydrate, lipid, nucleic acid or the like, which is capable of stimulating antibody production from B-cells.

The terms "ctxA" and "ctxB" refer to the gene sequences encoding the cholera toxin A and B subunits respectively.

The term "mucosal binding composition" is used herein to refer to compositions that include a mucosal binding polypeptide and an antigen from a pathogen capable of infecting a mammalian host through the mucosal membranes of the host. The term "mucosal binding polypeptide" is used herein to refer to polypeptide capable of attaching to the mucosal surfaces of a mammal.

There are a variety of mucosal binding polypeptides disclosed in the art. It is contemplated that the mucosal binding composition will include at least one mucosal binding polypeptide. Other polypeptides that associate with the mucosal binding polypeptide are also contemplated within the scope of this invention. The mucosal binding polypeptides of the present invention include, but are not limited to, bacterial toxin membrane binding subunits including, at a minimum, the B subunit of cholera toxin, the B subunit of the *E. coli* heat-labile enterotoxin, Bordetella

pertussis toxin subunits S2, S3, S4 and/or S5, the B fragment of Diphtheria toxin and the membrane binding subunits of Shiga toxin or Shiga-like toxins.

Other mucosa binding subunits contemplated within the scope of this invention include bacterial fimbriae protein including E. coli fimbria K88, K99, 987P, F41, CFA/I, CFA/II (CS1, CS2 and/or CS3), CFA/IV (CS4, CS5 and/or CS6), P fimbraiae, or the like. Other fimbriae contemplated within the scope of this invention include Bordetella pertussis filamentous hemagglutinin, vibrio cholerae toxin-coregulate pilus (TCP), Mannose-sensitive hemagglutinin (MSHA), fucose-sensitive hemagglutinin (PSHA), and the like.

Still other mucosal-binding molecules contemplated within the scope of this invention include viral attachment proteins including influenza and Sendai virus hemagglutinins and animal lectins or lectin-like molecules including immunoglobulin molecules or fragments thereof, calcium-dependant (C-type) lectins, selectins, collectins or *Helix pomatia* hemagglutinin. Plant lectins with mucosa-binding subunits include concanavalin A, Wheat-germ agglutinin, phytohemagglutinin, abrin and ricin.

The invention discloses the use of mucosal binding compositions to stimulate mucosal immunity to pathogens capable of infecting a mammalian host through the mucosal membranes of the host. In one preferred embodiment of this invention, a method for producing mucosal immunity to a pathogen is disclosed using the mucosal binding subunit of bacterial toxins. As one aspect of this embodiment, chimeric constructs of either the cholera toxin or the *E. coli* heat-labile enterotoxin are coupled to antigen obtained from pathogens capable of causing a disease. In another aspect of this embodiment, methods are disclosed for chemically coupling a mucosal binding polypeptide such as the mucosal binding portion of the B subunit of cholera toxin or of the heat labile enterotoxin of *E. coli* to antigen obtained from a pathogen capable of causing a disease.

It is additionally contemplated that the membrane binding compositions of this invention will be useful for stimulating secretory immunoglobulin. This secretory immunoglobulin can be used in diagnostic assays, such as ELISAs, immunoblots, or the like. In addition, it is contemplated that the binding compositions themselves can be used in assays to detect the presence of antibody in the sample to either the membrane binding polypeptide or to the foreign epitope. Moreover, the secretory antibody collected from experimental animals can be used in topical preparations against a specific pathogen or as a general component of a topical preparation or in additional studies to assess the character of secretory immunoglobulin.

There are a variety of methods available in the art for obtaining mucosal binding polypeptides. The polypeptides, or fragments thereof, can be isolated from nature or the polypeptides can be chemically synthesized or produced as a recombinant product from a prokaryotic or eukaryotic expression system. Those skilled in the art will be able to select and test a mucosal binding polypeptide for its ability to function as a carrier and to facilitate foreign antigen presentation to the immune system.

To select and determine whether a particular mucosal binding polypeptide will facilitate foreign antigen presentation to the immune system, one skilled in the art will begin by selecting a mucosal binding polypeptide from the literature or from other research sources. The mucosal binding protein can then be purified from nature or derived as a product of a recombinant expression system. Exemplary mucosal binding polypeptides have been

provided above. For recombinant protein expression, those skilled in the art of molecular biology will isolate nucleic acid fragments encoding this gene, using standard techniques known in the art, and incorporate these fragments into expression constructs. There are a wide range of eukaryotic and prokaryotic expression systems known in the art and it is recognized that the skilled artisan will be able to incorporate a nucleic acid fragment encoding a mucosal binding polypeptide into a variety of gene expression systems without undue experimentation. Exemplary gene expression constructs are provided here which are suited for gene expression in *E. coli* or *V. Cholerae*. Alternatively, the mucosal binding polypeptide can be isolated from nature using known purification techniques.

The recombinant or purified mucosal binding polypeptide is next tested for its ability to be bound by test antibody where the test antibody is known to recognize the naturally occurring mucosal binding polypeptide. Suitable tests for determining antibody recognition include ELISAs, immunoblots or other well known immunologic assays available in the art. Exemplary ELISA and immunoblot assays are provided in the Examples (see Example 4 and 6). These assays are well known and can be readily modified by those skilled in the art to identify other mucosal binding protein. Next, the candidate mucosal binding polypeptide is further tested for its ability to bind mucosa. Mucosal binding assays are known in the art. These assays can employ fixed mucosal tissue, primary mucosal cells placed in tissue culture, commercially available mucosal cell lines in culture, or assays involving mucosal cell membrane lysates or purified mucosal cell membrane protein. Mucosal tissue can be obtained by biopsy, and processed into tissue cell culture or as fixed tissue sections (from an autopsy specimen or as a punch biopsy). Mucosal cell lines are commercially available from a variety of sources. The following cell lines are available from the American Type Culture Collection (Rockville, Maryland). These include colon tissue (ATCC #'s CRL 1459, CRL 1539, CRL 1541 and CRL 1790), nasal septum (CCL 30), palate (CRL 1486), rectal cell lines (CCL 235, CCL 234), vulva-derived cell lines (HTB 88, HTB 117 and HTB 118), bronchal cell lines (CCL 208) and carcinoma cell lines of the mouth (CCL 17). Additionally binding assays can be performed in test animals. These assays may involve a reporter molecule such as a radioisotope, an enzyme linked to an antibody or to the mucosal binding composition to detect binding to the mucosa surface. Alternatively, the binding can be assayed using an antibody specifically recognizing the mucosal binding composition in an ELISA, a modification thereof, a western blot assay, or the like.

In a preferred series of embodiments of this invention, subunits or fragments of the *Vibrio cholerae* toxin are used to direct an immunogen to the mucosa. Embodiments are disclosed in the Examples, provided below, which detail the association of the mucosa binding polypeptide, the cholera toxin B subunit, to an immunogen derived from a non-viral pathogen causing a sexually transmitted disease.

Despite differences in the primary sequences of the proteins, there are a number of striking similarities between the nontoxic subunit of E. coli heat-labile enterotoxin (LTB) and the B subunit of cholera toxin (CTB). The LTB gene sequence is provided in Leong, J., et al. (*Infect. Immun.* 48:73-77, 1985 and see Tsuji, et al. *Microbial. Pathogenesis* 2:381-390, 1987). LTB, like CTB, can be secreted into the medium when *V. cholerae* cultures are transformed with expression vectors expressing recombinant LTB. Further, CTB and LTB bind to gangliosides on mucosal membranes (see Hirst, et al. *Proc. Natl Acad Sci USA* 83:9174-9178, 1984 and Schödel, et al. *Gene* 99:255-259, 1991). The Hirst, et al. reference indicates that CTB sequences can be replaced with LTB sequences

for recombinant expression. Thus, it is contemplated that CTB sequences can be replaced with LTB sequences and used in the methods and examples detailed below.

There are any number of constructs that one skilled in the art could prepare and test for their ability to promote the production of an antibody response to a non-viral pathogen causing a sexually transmitted disease where the mucosal binding polypeptide is derived from cholera toxin or the heat labile enterotoxin from *E. coli*. The non-limiting examples, provided below, include a variety of compositions employing mucosal binding polypeptide and include antigen from four different pathogens whose primary route of infection is through mucosa membranes.

The mucosal binding compositions of the present invention contain at least one antigen from a pathogen that enters its host through the mucosal membranes. This invention contemplates that the immunogen derived from the this pathogen can be selected from a variety of immunogens which are known to stimulate immune responses in a mammal susceptible to the disease caused by that pathogen. It is contemplated that the antigen includes at least one antibody stimulating determinant, preferably from a surface protein of the disease-causing pathogen. It is also contemplated that where the antigen is a polypeptide, it will contain at least one consecutive region of amino acids, preferably from an immunoaccessible domain of the surface polypeptide of the pathogen. In addition, the antibody stimulating polypeptide can optionally include one or more domains derived from the same or different proteins from the pathogen. These domains may include other antibody-stimulating amino acid sequences, multiple copies of these sequences or amino acid sequences that stimulate T-Cells or those that assist in the generation of an antibody response through the activation of T-Helper cells or other T-Cell populations. Further, it is contemplated that the antibody stimulating determinant can be repeated in tandem or separated by suitable linking sequences, or the like, to further stimulate the antibody response.

Thus, the first step for selecting an antigen contemplated in this invention is to identify a non-viral pathogen capable of infecting a mammal via entry through a mucosal membrane. Next, one can optionally determine whether those mammals who are infected with the pathogen develop an antibody response to the pathogen. This can be determined experimentally or based on the literature related to the particular pathogen.

As one example of a method for determining whether individuals who are infected with the pathogen develop an antibody response, serum samples are taken from a mammal and tested for the presence of antibody by contacting the serum sample with cell lysates containing pathogen, or alternatively with intact pathogen, and detecting binding of serum antibody to the sample. Assays useful for detecting serum antibody binding include enzyme-linked immunosorbent assays (ELISA), immunoblots such as Western blots, or the like. Such assays are well known in the art and are detailed in a variety of methodology texts including Harlow, et al. (Antibodies: A Laboratory Manual, Cold Spring Harbor Press, 1988). Optionally, those skilled in the art may elect to directly test for the presence of IgG or secretory antibody to the pathogen in a mammal. In these assays, secretory fluid is lavaged from the mucosal surfaces of a patient or test mammal and the volume is optionally reduced using any suitable reduction or concentration method well recognized in the art. This sample is then tested for the presence of pathogen-specific immunoglobulin, in general, or sIgA or IgG, specifically, using standard immunoassays well known in the art.

Alternatively, those skilled in the art can determine which protein or proteins stimulate a neutralizing immune response *in vitro* to the pathogen. Determinants stimulating immune responses are mapped to specific protein using the well known Western blot technique, or the like. The immune system stimulating determinants can be mapped to the protein using any number of strategies well known in the art. As one example of a method for mapping antibody stimulating determinants on a protein see Geysun, et al. and Miles, et al. ("Strategies for epitope analysis using peptide synthesis", *J. Immmunol. Methods* 102:259-274, 1987 and "Multiple Peptide Synthesis for the Systematic Analysis of B and T-cell Epitopes" *Parasitology Today* 5:397-400, 1989 respectively).

These screening regimes permit the identification of determinants capable of stimulating the immune system and are also well known in the literature for a variety of pathogens, including chlamydia. Methods are also well known in the scientific literature for selecting linear antigenic determinants that stimulate antibody production from B-Cells or selecting determinants that stimulate T-Helper cell activity. For strategies for identifying epitopes that stimulate T-Helper cell responses to *C. trachomatis* see Su, et al. (*J. Exp. Med.* 172:203-212, 1990). To identify epitopes that stimulate antibody production in *C. trachomatis* see Zhong, et al. (*Infect. and Imm.* 59:1141-1147, 1991).

Finally, it is contemplated that peptide mapping strategies, also well known in the art, can be used to identify a polypeptide that is capable of stimulating a neutralizing immune response to the pathogen. For methods for testing B-Cell stimulating determinants for their ability to stimulate neutralizing antibody *in vitro* see Zhang, et al. (*J. Immunol.* 438:575-581, 1987).

All of these steps permit one skilled in the art to identify candidate polypeptide sequences that can be linked to a mucosal binding polypeptide. These steps provide one strategy for identifying candidate polypeptide sequences which can be linked to a mucosal binding polypeptide, yet those skilled in the art will recognize that each step is not absolutely necessary for the identification of a polypeptide capable of generating an immune response.

Exemplary screening strategies are provided herein to enable those skilled in the art to determine whether a particular combination of polypeptide derived from a pathogen which enters its host through the mucosal membranes will stimulate antibody production in a mammal when linked to a mucosal binding polypeptide. These strategies are detailed in the examples below (see Examples 4 and 6).

As a first step for producing the composition of this invention, the mucosal binding protein is linked to the antigen selected from the pathogen. There are a variety of linking strategies contemplated within the scope of this application. For example, the antigen can be linked to the mucosal binding protein by chemical coupling or through a linking member, including lipid, carbohydrate or protein. Alternatively, the antigen can be synthesized by means of a gene construct either separately or together with the mucosal binding protein.

As specific examples of the use of the present invention to stimulate mucosal immunity to a pathogen capable of infecting its host through mucosal membranes, Example 1 details a preferred strategy for the chemical coupling of a membrane binding polypeptide to a determinant from a pathogen. In this example a fragment of the cholera toxin protein B subunit was expressed using the method of Lebens, et al. (*BioTechnology* 11:1574-1578, 1993) and chemically coupled to an immunogen derived from the major outer membrane protein of *C. trachomatis*.

In one preferred embodiment employing chemical coupling, the polypeptide derived from the major outer membrane protein is the VDIV peptide (SEQ ID NO:1) corresponding to a portion of the fourth variable domain of the major outer membrane protein of *C. trachomatis*. In another preferred embodiment, the polypeptide is a linear chimeric polypeptide that comprises the A8 domain and the VDIV domain from the major outer membrane protein of C. trachomatis (SEQ ID NO:2). Both the A8 and the VDIV domain as well as the chimeric peptide are described by Su, et al. (*Vaccine* 11:1159-1166, 1993).

As another embodiment of this invention, and as an example of alternative linking strategies, a polypeptide from a protein derived from a non-viral pathogen causing a sexually transmitted disease is expressed as a recombinant protein with a subunit or fragment of a mucosal binding protein. As one example of the recombinant expression of this complex, the mucosal binding protein is a portion of the cholera toxin and the antigenic determinant is derived from *C. trachomatis*.

It is contemplated that there are a variety of construct designs that could be used to express protein having a portion of the cholera toxin and a polypeptide from a protein derived from a pathogen capable of infecting its host through mucosal membranes. For example, the antigenic determinant or determinants from the pathogen could be placed at the amino or carboxy terminus of the B subunit, at the amino or carboxy terminus of the A subunit, or at the amino terminus of a portion of the A subunit. In a preferred example, the A subunit is operatively linked to the B subunit. In another contemplated embodiment, detailed in the Examples below, the antigenic determinant is incorporated in frame into an internal portion of either the A or B subunit, or a portion thereof.

It is contemplated that these constructs, provided in the examples below, could also be derived from the B subunit of the heat labile enterotoxin of *E. coli* without undue experimentation. It is further contemplated that variations in the plasmids are also within the scope of this invention. For example, this work has also demonstrated that the high copy number derivative pML-LCTB*tac*2 has an identical restriction pattern to pML-LCTB*tac* but incorporates the pUC19 origin of replication (see Lebens, et al. *Biotechnology, supra*). Thus, the use of this plasmid is also contemplated within the scope of this invention.

In one construct detailed in Example 2, a chimeric protein is produced that includes a polypeptide linked at its carboxy terminus to the amino terminus of the B subunit (CTB) from cholera toxin. As another example to indicate that this construct is similarly useful for producing stimulating antibody to other antigens, the HIV epitope IQRGPGRAFV is incorporated into the amino terminus of the B subunit.

In another example of this embodiment, detailed in Example 3, the antigen from the pathogen is incorporated onto the amino terminus of a polypeptide which is a fusion protein of a fragment of the A subunit of cholera toxin fused to CTB and expressed in either *V. cholera* or *E. coli*. In one aspect of this embodiment, the antigenic peptide is the VDIV sequence from the major outer membrane protein of *C. trachomatis* and in another preferred embodiment the antigenic peptide is the A8 sequence from the major outer membrane protein. In a third aspect of this embodiment, the peptide is a chimeric protein containing both the antigenic peptide sequences of A8 and VDIV separated by a linker (see Figure 6). It is also contemplated that this same linker introduced into, for example, pML-LCTBλ7, would facilitate the construction of multiple copies of either epitope. Alternatively, the linker, or other

linkers, could be used to insert the A8 and VDIV sequences into other constructs or the linker could facilitate the transfer of inserted sequences between vectors. It is further contemplated within the scope of this embodiment that the order of the antigenic sequences can be switched and that multiple determinants of A8 or VDIV can be linked either in tandem or within the recombinant construct.

The literature describes a variety of antigenic peptide sequences for *C. trachomatis* that are serovar specific, subgroup specific or are broadly conserved among serovars. Thus, it is also contemplated within the scope of these embodiments that other antigenic peptides from the major outer membrane protein or from other proteins of *C. trachomatis* would also be useful in stimulating antibody production along the mucosal surfaces.

Jobling, et al. (*Infect. and Imm.* 60:4915-4924, 1992) have produced fusion proteins in which entire bacterial proteins were linked via the amino terminus to the A subunit fragment, CTA2. Whereas Jobling, et al. demonstrated that chimeric toxin could be produced from a cholera toxin construct, both portions of the chimera were bacterial protein and the signal sequences for chimeric expression were derived from the inserted bacterial protein. The present invention demonstrates that antigenic sequences which are foreign to the bacteria can be inserted into CTA2. The CTA2 fusion proteins with foreign antigenic sequences were coexpressed with CTB from *V. Cholera* to give assembled products which were excreted extracellularly and were detectable in a GM1-ELISA. The gene products produced by Jobling et al. were not excreted extracellularly.

In a third example employing genetic constructs, contemplated within the scope of this invention and detailed in Example 4, the polypeptide is incorporated, in frame, into an internal portion of the mucosal binding polypeptide, preferably at an immunoaccessible site. In one example of this strategy, it is contemplated that a short foreign polypeptide is introduced into an internal region of the cholera B subunit in place of CTB amino acids 56-63. In one embodiment, this peptide includes the VDIV epitope, LNPTIAG, and in another embodiment, illustrating that this strategy can be used for other peptide sequences, the peptide is the HIV neutralizing epitope IQRGPGRAFV (aa 309-318 of HIV-1 isolate HTLV-IIIB).

Six additional plasmids were constructed, encoding internal HIV::CTB hybrid proteins with ten to fourteen amino acids from the V3 loop of gp120 genetically inserted at different positions between amino acids 52 and 65, with deletions of different CTB (Cholera toxin B subunit) amino acids (See Table 1). The plasmids which encoded proteins with peptides inserted between 55 and 64, 53 and 64 or 56 and 57 in CTB gave rise to the synthesis of proteins which reacted with CTB-specific monoclonal antibodies (mAb) and bound to GM1 gangliosides. This indicated that insertions at these sites did not alter the functional conformation or the receptor binding properties of native CTB.

In yet another example, the peptide is a portion of the pre-S(2) protein from Hepatitis B and in a further example the peptide is one of two peptides from the ST_a protein from enterotoxigenic *E. coli*. Plasmids were constructed encoding CTB hybrid proteins which had either an eleven amino acid peptide form Hepatitis B Virus (HBV) pre-S(2) (SEQ ID NO: 28) or one of two peptides (SEQ ID NO:30 and SEQ ID NO: 31, see Table 1) related to the heat-stable toxin (ST_a) of enterotoxigenic *E. coli*. This work demonstrated that an internal permissive site could be used to insert peptides of several different amino acid compositions.

Epitope-specific antibodies were used in GM1-ELISA and immunoblot assays to screen the protein hybrids for their conformational integrity, for their ability to bind to membrane determinants or to be recognized by the foreign epitope specific antibody. Example 4 provides data relating to this screening. All of the CTB:foreign antigen products induced low levels of serum antibodies in mice against the full length foreign protein. In addition the products stimulated strong serum antibody responses against CTB.

Data is provided in Example 4 to indicate that the CTB::foreign antigen hybrids of the present invention retain all of the important characteristics of native CTB, such as folding, pentamerization, extra-cellular secretion when produced in *V. cholerae* and GM1-binding. Moreover, many of the inserted peptide constructs were resistant to cleavage by *V. cholerae* proteases. The data in this example indicated that the CTB::foreign antigen hybrids reacted with monoclonal antibody directed against the foreign antigen, both in the denatured and non-denatured forms of the protein. This indicated that the substitution of eight amino acids from a region in the native molecule with amino acids from an unrelated protein, such as gp120, VDIV, pre-S(2), and ST, produces a construct with foreign antigen that is accessible to the immune system of its host.

It is also contemplated within the scope of the invention that the environment of the inserted peptide can be modified to improve the immunogenicity. For example, it is contemplated that flanking residues can be added to the inserted peptides or that the position of the insert can be shifted within the insert permissive region of the CTB sequence and that these modifications do not limit the scope to this invention.

Once the mucosal binding complex of binding protein and antigen has been generated it will be helpful to test the integrity of the complex using antibody to the foreign antigen and antibody to the mucosal binding protein. Methods for detecting hybrid protein expression are provided in Examples 4 and 6. *In vitro* assays testing mucosal binding are useful for demonstrating the intactness of the complex. Such assays include binding assays to intact mucosal epithelium cell cultures or mucosal membrane sections. If the mucosal receptor is known, specific ELISAs or receptor/ligand chromatographic assays, or the like, can be used to ascertain mucosal binding.

Next, it is contemplated that the complex will be formulated as a mucosal binding composition that optionally includes physiologic buffers, additional adjuvant, or the like, to facilitate the production of an immune response in a mammal. The composition is then introduced into a mammal either vaginally, orally, rectally, nasally, intramuscularly, intraperitoneally or intravenously. Sera or mucosal immune responses are monitored in the experimental mammal or in clinical trial by ELISA, immunoblot, or by western blot to detect antibody to either the mucosal binding polypeptide or the foreign polypeptide. It is contemplated that antibodies in serum or antibody in mucosal secretions can be assayed for the presence of IgA, IgG or total antibody reactivity to a particular peptide, polypeptide or pathogen preparation using assays well known in the art. The mucosal secretions are harvested as a lavage, an aspirate or as a wash preparation. Alternatively, the mucosal secretions are collected through wicking action using an absorbent pack, such as a tampon, an absorbent plug, or the like. Similarly, biopsy of mucosal tissue during the testing phases of this invention will be used to assay, by histopathology or by cell activity, for immune responses specific to the mucosal binding polypeptide. For exemplary methods for preparing biopsy tissue see Eriksson, et al. or Quiding, et al. ("Perfext: A Simple Perfusion-Extraction Procedure for Quantitative Analyses of

Antibodies and Cytokines in Distinct Anatomical Compartments" Abstract presented at the 12th European Immunology Meeting. June 14-17, 1994 and *J. Clin. Invest.* 88(1):143-148, 1991 respectively). Strategies for detecting IgG and IgA in a mammal are known in the art. These include the measurement of antibodies in serum or mucosal secretions using ELISAs, immunoblots, or the like.

This invention details methods for introducing the compositions of the present invention into a mammal for stimulating antibody production. Methods are provided for stimulating vaginal, oral and rectal immune responses (see Examples 7-9).

Results detailed in Example 7 indicated that immunization of mice with the intrachain CTB::HIV hybrid protein gave rise to very strong serum antibody responses to the CTB moiety. Antibody to the foreign epitope was also observed. Example 10 indicates that the combination of a mucosal binding polypeptide with a foreign antigen from a pathogen produced mucosal antibody specific to the pathogen.

All references cited herein are expressly incorporated by reference in their entirety. Particular embodiments of the invention are discussed in detail below and reference has been made to possible variations within the scope of the invention. There are a variety of alternative techniques and procedures available to those of skill in the art which would similarly permit one to successfully perform the intended invention.

EXAMPLE 1
Chemical Coupling of Polypeptide to a Membrane Binding Protein

In this example, Cholera toxin B subunit (CTB) was produced in a mutant strain of *Vibrio cholerae* deleted of the cholera toxin genes and transfected with a plasmid encoding CTB (as disclosed by Lebens, et al. *supra*). In this expression system, CTB was recovered as secreted protein in yields at or above 1 g/l. Bacterial cultures were centrifuged at 8000 rev per min for 220 min and the supernatants were adjusted to pH 4.5 with dilute HCl. After precipitation with hexametaphosphate (final concentration of 2.5 g/l) for 2 hr at 23°C followed by centrifugation at 8000 rev per min, the pellets were dissolved with 0.1M sodium phosphate buffer, pH 8.0 and dialyzed against 0.01 M phosphate-buffered saline (PBS), pH 7.2. The dialysate was then centrifuged at 15,000 rev per min to remove insoluble material and the supernatant was further clarified by filtration through 0.22 μm filters (Millipore, Bedford, MA). CTB was purified by standard gel filtration chromatography through columns of Sephadex G-100 (Pharmacia, Upsala, Sweden).

The polypeptides were covalently conjugated to CTB using N-succinimidyl(3-[2-pyridyl]-dithio) propionate (SPDP, Pharmacia) as a bifunctional coupling reagent according to the manufacturer's instructions. CTB was derivatized with SPDP at a molar ratio of 1:5 in 0.1M phosphate buffer/0.1M NaCl pH 7.5. After incubation at 23 °C for 30 min., excess SPDP was removed by gel filtration through Sephadex G-25 columns (Pharmacia) and eluted with PBS. The absorbance of the modified protein was measured at 280 nm. To estimate the degree of substitution with 2-pyridyl disulphide residues, the absorbance at 343 nm of 100 μl protein solution in 400 μl was measured after incubation with 50 μl dithiothreitol (0.1M) for 15 minutes (molar extinction coefficient at 343 nm = 8.08 x 10^3 $M^{-1}cm^{-1}$). This concentration was equivalent to the concentration of 2-pyridyl disulphide residues in the protein. Since the 2-pyridyl disulphide groups contribute to the absorbance at 280 nm, a correction was applied to the

calculation of protein concentration: A_{280} due to the protein = A_{280} - (B x $5.1x10^3$) where B is the molar concentration of pyridine-2-thione released. From these results the number of moles of 2-pyridyl disulphide substituted per mole of protein were calculated. The substitution ratio obtained with these conditions ranged between 2-3 moles of SSPY per mole of CTB pentamer.

Peptides and SPDP-derivatized CTB were mixed at a ratio of 5 mol peptide/1 mol SSPY and incubated for 24 hr at 23°C. The resulting CTB-peptide conjugates were purified by gel filtration through columns of Sephadex G-25. Conjugates were purified over GM1-columns of Sephadex G-25. Purified conjugates were shown to retain GM1-binding capacity and to retain both CTB and peptide specific serological reactivities by means of a solid phase ELISA using GM1 as a capture system and enzyme-labelled antibodies to CTB or to the foreign polypeptide sequence coupled to CTB. An exemplary ELISA using GM1 is provided in Examples 4 and 6, below.

In this embodiment, three different peptides were conjugated to CTB. The first, Peptide 166 (SEQ ID NO:13, A8-VDIV), is the colinear peptide disclosed by Su, et al. (*Vaccine* 11:1159-1166, 1993). It contains a single cysteine residue. Peptide 172 (SEQ ID NO:14) has a free extra cysteine coupled to the amino end and Peptide 173 (SEQ ID NO:15) has a free extra cysteine coupled to the carboxyl end of the A8-VDIV sequence.

The CTB/A8-VDIV complexes were introduced into C57 BL/6J female mice (obtained from the Animal Care Facility of the Department of Medical Microbiology and Immunology, University of Goteborg). Mice were 6-8 weeks of age. The immunization protocol for generating mucosal antibody is provided in Example 8.

EXAMPLE 2
Genetic Fusion of Foreign polypeptide to the amino terminus of CTB

In one embodiment, the starting plasmid was pPL-lambda obtained from Pharmacia AB, Sweden. The generation of the pMLλ plasmids is diagramed in Figure 1. pPL-lambda was digested with SmaI and BamHI. Digested DNA was resolved on an agarose gel and a 1,217 bp fragment carrying the lambda promoter region was recovered by band-extraction. More of the same plasmid was digested with BamHI and PvuII and again resolved on an agarose gel. This time a 2,305 bp BamHI/PvuII fragment carrying the pBR322 origin of replication and the ampicillin resistance gene was recovered by band extraction. The two extracted bands were ligated together and ligated DNA was transformed into *E. coli* strain N4830-1, the Gal-P2 transductant of N4830 (Pharmacia, Sweden). In the resulting plasmid, pMLλ1, the orientation of the phage lambda DNA within the plasmid has been reversed and 1,689 bp of pBR322 DNA has been removed.

pMLλ1 was digested to completion with BamHI and subsequently partially digested with HaeIII. The digested DNA was religated and used to transform *E. coli* strain N4830-1. Transformants were screened on the basis of restriction analysis for plasmids in which the lambda N gene and tL 1 terminator had been removed. The resulting plasmid was pMLλ2 and carried the promoter λP, with unique downstream restriction sites BamHI, SmaI and EcoRI that could be used for cloning of recombinant genes. The powerful *trp*A transcription terminator (derived from the *trp*A cassette, Pharmacia) was introduced between the unique EcoRI and AatII sites within pMLλ2. At the

same time, an additional unique XhoI site and a HindIII site that could be used in cloning procedures were introduced into the plasmid (see Figure 1).

The unique NdeI site within the plasmid was removed by digestion with NdeI, blunt end repaired with the Klenow fragment of DNA polymerase and religated. The SspI site in the vector was removed by insertion of an EcoRI linker which generated BspEI sites on either side of the introduced EcoRI site. The resulting plasmid was digested with BspEI and religated. This plasmid was used for recombinant expression in *E. coli*.

Fusions of peptides to the amino terminus of CTB were generated by inserting synthetic oligonucleotides between unique SacI and SspI sites in the expression vector pML-LCTBλ7 (see Figure 3). For example, the HIV RP335 peptide was placed at the amino terminus of CTB in pML-LCTBλ7 between the SacI and SspI sites using two oligonucleotides: SEQ ID NO:9 and SEQ ID NO:10. In a second construct, the insertion was placed within the structural gene at position +3 of the CTB sequence generating a HpaI site and at the same time destroying the SspI site. Oligonucleotides corresponding to SEQ ID NO:11 and SEQ ID NO:12 were used for this vector construction. The *ctx*B gene in this vector is under the control of the inducible λP_L promoter and was constructed essentially as described previously (Lebens, M. et al *BioTechnology* 11:1574-1578, 1993). The vector was chosen in order to allow the construction of genetic fusions, the products of which may be deleterious to the host cells since the inducible system only allows expression under inducing conditions. Thus, using these constructs, cultures can be maintained under conditions in which expression is not switched on and the recombinant protein does not accumulate to harmful levels. Expression at high levels can be induced for a short time immediately before the cells are harvested at which point survival of the culture is no longer an issue.

In another embodiment, a second construct was produced that employed pML*tac* derivative plasmids. This permitted expression of the resulting protein fusions in *V. cholerae*. The starting plasmid in this case was the expression vector pKK223-3 obtained from Pharmacia AB (Sweden). In order to obtain the plasmid used as the basis of the overexpression system for CTB and its derivatives, the 1689bp PvuII/BamHI fragment was removed. This was achieved by digestion of a plasmid into which the *ctx*B gene had been inserted with PvuII and BamHI, followed by blunt end repair of the plasmid with Klenow enzyme and subsequent religation. This procedure regenerates the BamHI site shown in Figure 2.

In the course of exchange of different *ctx*B derivatives between the two expression systems, the *rrn*B T1T2 transcription terminator was replaced with the *trp*A terminator described above.

A further development of the vector was to introduce the *lac*I gene upstream of the *tac* promoter to make the vector produce cloned gene products in an inducible manner. This was done using PCR. The P*tac*-based expression plasmids of this invention (see Figure 2) were amplified using SEQ ID NO:16 and SEQ ID NO:17. The *lac*Iq gene was obtained by PCR fragment amplification of expression plasmid pMMB66 (Furste, et al. *Gene* 48:119-131 1986) using SEQ ID NO:18 and SEQ ID NO:19. The *lac*Iq gene was introduced between BamHI and BglII restriction sites so that it could be removed easily to generate a plasmid giving constitutive expression from the *tac* promoter (see Figure 2).

In a third embodiment, the same gp120 peptide was fused to the N-terminus of CTB by inserting complementary synthetic oligonucleotides encoding the peptide between the *Sac*I and *Xma*I restriction sites at the junction between the leader peptide and the mature CTB in the expression plasmid pJS752-3 (Ap^R), a derivative of pJS162 (Sanchez, et al. *Proc. Natl. Acad. Sci. USA* 86: 481-485, 1989). This plasmid is essentially the same as pML-LCT*Btac* except that the EcoRI/HindII fragment carrying the recombinant ctxB gene is derived from pJS162. The parent plasmid is the expression vector pKK223-3 (Pharmacia). Plasmid JS752-3 carries the gene encoding CTB under *tac* promoter control. In plasmid pJS54 (see Example 4, below), an oligo (formed from the hybridization and ligation of oligonucleotides corresponding to SEQ ID NO:20 and SEQ ID NO:21) was inserted between unique SacI and SmaI sites in pJS752-3.

The recombinant *V. cholerae* strain producing the N-terminal CTB::HIV hybrid protein was shown to secrete the chimeric protein into the culture medium using the GM-1 ELISA provided in Example 4. The cultured cells secreted protein reactive with mAb F58/H3-anti-gp120 when analyzed in a GM1-ELISA. The GM1-ELISA was useful as a tool to quantitate the amount of expressed protein and to monitor the extent of proteolytic degradation of the added peptide. Culture conditions required to produce the constructs are provided in Example 5.

In another embodiment, another construct with an amino terminus substitution was made in which the gp120 epitope was placed at the N-terminus of the CTB protein with two extra amino acids, corresponding to the two first amino acids of CTB, placed N-terminally to the gp120 peptide, and this extended epitope was linked directly to the complete mature CTB. The resulting plasmid, pCB2gp309-318, was expressed in *V. cholerae* JS1569 (strain 644). When cultured at 37°C, a protein which bound to GM1 reacted with the CTB-specific mAb LT39 and was produced within the periplasm. Unlike native CTB, it was not actively secreted into the medium. However, the protein produced by strain 644 reacted with mAb P4/D10 increasingly with time. The fused gp120 peptide (SEQ ID NO: 29) in this protein was more resistant to proteolytic degradation than the construct lacking the N-terminal amino acids of CTB. The plasmid pCB2gp309-318 was also expressed in *E. coli* HB101 (strain 504) and could be purified from the periplasmic space by osmotic shock lysis of the cells followed by precipitation with 80% ammonium sulfate and extensive dialysis against PBS. This protein ran at a higher molecular weight than CTB in SDS-PAGE, indicating the formation of aggregates, but the protein reacted well with both mAb P4/D10-anti-gp120 and with mAb LT39 and CT6 against pentameric and monomeric CTB.

EXAMPLE 3
Genetic Fusion of Foreign polypeptide to the amino terminus of CTA-2

The plasmid used for the expression of CTA-2 was constructed so that the CTA-2 fusion and CTB were co-expressed to obtain assembly of a holoprotein *in vivo*. The *ctx*A2 and *ctx*B genes were obtained from PCVD30 (See Kaper, J. et al. *BioTechnology* 2, 345-349, 1984) as an XbaI/HindIII fragment which was cloned into pUC19 (Yanisch-Perron, C., et al. *Gene* 33, 103-109, 1985). The *ctx*A ribosome binding site and signal peptide sequence were reintroduced by the insertion of synthetic oligonucleotides between the XbaI site and a unique EcoRI site upstream from it in the vector (see Figure 4). The resulting EcoRI/HindIII fragment carrying the *ctx* genes contained

unique SacI and XbaI sites between which synthetic oligonucleotides can be inserted to generate amino terminal fusions between *ctx*A2 and the added epitope of interest. This fragment was then transferred to each of the expression plasmids illustrated in Figures 2 and 3. In each case, plasmid-derived expression of CTB could be demonstrated. Plasmids carrying the *ctx* genes under the control of the *tac* promoter were maintained in *V. cholerae* since the levels of CTB generated in this example were too high to be tolerated by *E. coli*. This construct lacks the recognition site for the proteolytic cleavage involved in the maturation of CTA.

The antigens used in this work were those from *Chlamydia trachomatis* strains identified by Su and Caldwell (Su, H. et al. *Vaccine* 11, 1159-1166, 1993). These include a T-cell epitope A8 SEQ ID NO:6, situated within a 25 amino acid peptide from serovar A MOMP and a B-cell epitope VDIV SEQ ID NO:1, from serovar B MOMP containing 17 amino acids. Within the VDIV sequence a septapeptide sequence has been mapped as an epitope reacting with the monoclonal antibody DIII-A3. The amino acid sequences of the two peptides are shown in Figure 5 together with the synthetic nucleotide sequences used for the generation of gene fusions with CTB. The oligonucleotide corresponding to VDIV and the oligonucleotides corresponding to A8 are identified.

The first of the fusions were made to the amino terminus of CTA2. The synthetic oligonucleotides corresponding to the foreign antigen were cloned into the vectors. It was possible to express assembled protein complexes in which the VDIV peptide was associated with CTB and detectable in a GM1 ELISA assay using Mab DIII-A3 as the primary antibody. In this case the assembled protein was produced in *E. coli* under the control of the λP1 promoter. In *V. cholerae*, assembled protein was detected in the periplasm when the construct was under control of the *tac* promoter. The A8 peptide was cloned independently into similar vectors.

In order to produce a fusion between the A8 and VDIV peptides attached to CTA2, the linker shown in Figure 6 was inserted into the SacI site of plasmid pPJ-VDIVλ using SEQ ID NO:7 and SEQ ID NO:8. This allowed the addition of the A8 sequence to the amino end of the VDIV sequence by cloning of a BglII/XbaI fragment from pPJA8λ into BglII/NheI digested pPJVDIVλ1. pPJA8λ is a CTA2 fusion vector into which the oligo encoding the A8 T-cell epitope was inserted.

EXAMPLE 4
Insertion of a Neutralizing Antigen in a Surface Exposed Internal Region of the Cholera Toxin B-Subunit

In this example, novel intrachain CTB fusion protein were prepared with foreign peptide inserted into an internal region of CTB. The resulting chimeric protein retained important functional characteristics of the native CTB including: 1) pentamerization; 2) GM1 ganglioside receptor binding; and 3) resistance to proteolytic degradation during production of the protein in *Vibrio cholerae*. The inserted epitopes were detected with antibody known to bind the epitopes using ELISA and immunoblot assays to demonstrate that the epitope was present and accessible on the surface of the protein. Immunization of mice with the test hybrid protein elicited antibody responses to the mucosal binding polypeptide and the inserted antigen.

In one example, the foreign polypeptide was inserted at the position of an internal loop structure between β4 and α2 in CTB, extending with a few residues into the α-helix, as predicted by a comparison of the resolved crystal structure of LTB (Sixma, et al. *Nature* 351:371-377, 1991). Here, the VDIV fragment TTLNPTIAGAG is incorporated into the internal CTB site using the two restriction endonuclease recognition sites *Kpn*I and *Msc*I. Oligonucleotides corresponding to SEQ ID NO: 22 and SEQ ID NO:23 are hybridized, digested with the restriction enzymes *Kpn*I and *Msc*I, purified and ligated to plasmid pCB56-64.

In a second example, expression vectors were constructed having HIV-1 epitopes positioned internally in-frame within CTB, the CTB expression plasmid pML-LCTB*tac* (Ap^R) was mutagenized using the polymerase chain reaction (PCR), employing a modified protocol of that disclosed by Schödel et al. ("Hybrid hepatitis B virus core/pre-S particles expressed in attenuated *Salmonellae* for oral immunization." In: Brown, F., et al. (Eds), Vaccines '91. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1991, pp. 319-325.). In the first construct, the oligonucleotides used in the PCR reactions are provided as SEQ ID NO:3 and SEQ ID NO:4. These primers incorporated a sequence encoding ten amino acids from the central portion of the V3 loop in HIV gp120 between residue 55 and 64 in CTB. Two unique restriction enzyme sites, *Bss*HII and *Kpn*I, were also introduced, with the oligonucleotide primers, into the final plasmid pCB55-64gp309 (see Figure 7).

The PCR reaction was run in the presence of 1.5 mM $MgCl_2$ and 1.25 mM deoxynucleotides under the following reaction conditions: denaturation at 94°C for 1 min, annealing of primers at 65°C for 2 min and elongation of DNA by *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, Indiana) at 72°C for 4 min. The reaction was repeated for 30 cycles, increasing the elongation step by 2 sec with each cycle. To complete all synthesized DNA strands, a final incubation step at 72°C for 10 min was performed. After phenol/chloroform extraction, the PCR product was digested with *Bss*HII (Boehringer Mannheim) according to the manufacturer's instructions, phenol/chloroform extracted again and religated using T4 DNA ligase (Pharmacia, Upsala, Sweden) at 16°C for 3 h. The ligated plasmid was electroporated into *V. cholerae* (strain JS1569, *ctxA*$^-$, *ctxB*$^+$) by the method of Lebens et al. (*supra*). The DNA sequence was confirmed by the dideoxy chain termination method of Sanger et al. (*Proc. Natl. Acad. Sci. USA* 74:5463-5467, 1977).

The CTB region was selected for substitution because it had been reported that this region reacted with antibodies recognizing primary protein structure rather than CTB conformational epitopes (Jacob et al., *EMBO J.* 4:3339-3343, 1985 and Kazemi, et al., *Mol. Immunol.* 29:865-876, 1991). In addition, based on the crystal structure of LTB, a substitution in this region would presumably not affect the beta-sheet or alpha-helix structures essential for the correct folding of the molecule. Further, based on the crystal structure of LTB, a substitution in this area would not affect the assembly of pentamers (see Sixma et al., 1991, *supra*). Finally, a substitution is this area would likely not interfere with the site responsible for GM1 receptor binding on the mucosal surface.

The gp120 peptide used for these studies was taken from amino acids 309-318, having the sequence IQRGPGRAFV (SEQ ID NO:29), representing a portion of the V3 loop of HIV-1 isolate HTLV-IIIB. The sequence numbering of gp120 is based on the Los Alamos database sequence for gp120 (Los Alamos National Laboratories, Los Alamos, New Mexico). The sequence contains a principal neutralizing B-cell determinant of HIV-1. The sequence

GPGR within the peptide is conserved between several HIV-1 isolates and the peptide is of great interest in the development of a peptide-based vaccine against HIV-1 (Javaherian et al., *Proc. Natl. Acad. Sci. USA* 86:6768-6772, 1989). The gp120 peptide replaced amino acids 56-63 of the recombinant CTB, thus adding two amino acids to the net number of residues in the protein.

Polyacrylamide gel electrophoresis (PAGE) and immunoblot analyses of partially purified proteins from culture supernatants of *V. cholerae* carrying the plasmid pCB55-64gp309 revealed that the internal hybrid protein was synthesized and actively secreted from the cell into the culture medium like native CTB, accumulating to approximately 5-15 mg per liter medium. Cultures were grown in 50ml modified syncase medium in 250 ml Erlenmeyer flasks shaken at 250 rpm overnight at 37 °C. These plasmids can also be grown in *E. coli* without adjustments in vector construction.

A number of additional plasmids were prepared which incorporated foreign antigen into the CTB sequence between positions 54 and 64 of the CTB protein. The features of the different plasmids are listed in Table 1 (see page 38). pCB55-64gp309 (Ap) refers to the CTB:HIV hybrid protein described in the preceding paragraphs of this example. The constructs of Table 1 were prepared using the methods described above. Plasmid pCB55-64gp309-318 used SEQ ID NO: 32 and SEQ ID NO:33 as oligonucleotides to incorporate the gp120 epitope into the CTB protein. Plasmid pCB56gp309-318 used SEQ ID NO: 34 and SEQ ID NO: 35; pCB52-58gp309-318 used SEQ ID NO: 36 and 37, pCB53-64gp309-318 used SEQ ID NO: 38 and SEQ ID NO: 39, pCB53-64gp307-318 used SEQ ID NO: 40 and SEQ ID NO: 41, pCB55-64gp309-322 used SEQ ID NO: 42 and SEQ ID NO: 43, pCB55-64STdeca used SEQ ID NO: 44 and SEQ ID NO: 45, pCB55-64 st, used SEQ ID NO: 46 and SEQ ID NO: 47, pCB55-64ps133-143 used SEQ ID NO: 48 and SEQ ID NO: 49, and pCB56ps133-143 used SEQ ID NO: 50 and SEQ ID NO: 51.

Plasmids pCB53-64gp309-318 and pCB53-64gp307-318 were made by the cloning of complementary synthetic oligodeoxynucleotides (oligos) between the *Kpn*I and *Bss*HII sites in pCB55-64gp309-318 with the introduction of a *Bam*HI site in the latter. In order to construct the plasmid pCB55-64gp308-322, complementary synthetic oligos encoding amino acids 314-322 from gp120, carrying a *Bss*HII site, were cloned in between the unique *Nsi*I and *Msc*I sites flanking the peptide inserted between 55 and 64 in the plasmid pCB5-64gp12. A *Bss*HII/*Hind*III fragment of around 1 kb, encoding amino acid 315-322 of the inserted epitope together with amino acids 64 to 103 of the gene encoding CTB (*ctxB*) and sequence up to the terminator sequence was obtained from the intermediate plasmid and subcloned between the *Bss*HII and *Hind*III sites in plasmid pCB55-64gp309-318.

The other HIV::CTB gene fusions were made by oligo directed PCR mutagenesis of the expression vector pML-LCTB*tac*, introducing nucleotides coding for amino acid 309-318 from gp120, and containing a *Bss*HII site, either as a straight insertion at position 56, resulting in plasmid pCB56GP309-318, or with deletions of the CTB amino acids 56-64 (pCB55-65GP309-318) or amino acids 53-57 (pCB52-58gp309-318).

To make the ST::CTB plasmids, two pairs of synthetic oligos were synthesized which encode either a ST_a-related decapeptide containing a neutralizing B-cell epitope, or the whole ST_a of 19 aa, with a *Kpn*I site at the 5'- and a *Msc*I site at the 3'-end. A *Sph*I site was introduced with the oligos, which were cloned into the plasmid pCB55-64gp12, to obtain the plasmids pCB55-64ST_{dec} and PCB55-64ST_a respectively.

The plasmids encoding the HBV::CTB hybrid proteins were also constructed by oligo directed PCR mutagenesis of pML-LCTB*tac*, introducing DNA encoding amino acids 133-143 from pre-S(2) together with an *Xho*I site, either between amino acids 55 and 64 of CTB, yielding the plasmid pCB55-64ps133-143, or as a straight insertion after position 56 in the plasmid pCB56ps133-143.

All hybrid genes were sequenced by the dideoxy chain termination method of Sanger et al. (1977).

Table 1. Properties of the different plasmids and the corresponding hybrid proteins

Plasmid	Recombinant *V. Cholerae* strain number[a]	CTB aa deleted	Aa in inserted epitope	Aa sequence of inserted epitope	Unique restriction enzyme sites introduced with the insert
pCB55-64gp309-318[b]	407	56-63	gp120 309-318[c]	IQRGPGRAFV	*Bss*HII, *Kpn*I
pCB55-65gp309-318	408	56-64	gp120 309-318	IQRGPGRAFV	*Bss*HII, *Kpn*I
pCB56gp309-318	439	-	gp120 309-318	IQRGPGRAFV	*Bss*HII
pCB52-58gp309-318	440	53-57	gp120 309-318	IQRGPGRAFV	*Bss*HII
pCB53-64gp309-318	460	54-63	gp120 309-318	IQRGPGRAFV	*Bss*HII, *Kpn*I
pCB53-64gp307-318	586	54-63	gp120 307-318	IRIQRGPGRAFV	*Bss*HII, *Kpn*I, *Bam*HI
pCB55-64gp309-322	550	56-63	gp120 309-322	IQRGPGRAFVTIGK	*Bss*HII, *Kpn*I
pCB2gp309-318	644 (504[d])	-	gp120 309-318	IQRGPGRAFV	*Bss*HII
pJS54	285	-	gp120 309-317	IQRGPGRAFPGYAHG[e]	*Xma*I
pCB55-64STdeca	551	56-63	ST decapeptide	CAELCCNPAC	*Sph*I
pCB55-64st$_1$	557	56-63	ST 1-19	NSSNYCCELCCNPACT GCY	*Sph*I
pCB55-64ps133-143	395	56-63	pre-S(2) 133-143	DPRVRGLYFPA	*Xho*I
pCB56ps133-143	549	-	pre-S(2) 133-143	DPRVRGLYFPA	*Xho*I

a) The maternal strain is *V. cholerae* JS1569

b) The nomenclature used for the plasmids is as follows: CB indicates that the maternal protein is CTB; the first number indicates the position in CTB of the inserted peptide (a straight insertion without deletions is noted with the aa which the insert is placed after the two numbers (eg. 55-64) indicate that the aa between the numbers are deleted); the next two letter describes the origin of the inserted aa, gp, for gp120, ps for pre-S(2) and ST for STa, and the last numbers or letters indicated the aa inserted

c) Numbering of gp120 aa according to the Los Alamos database

d) Strain number 504 is an *E. coli* HB101 strain carrying the plasmid pCB2gp309-318

e) Amino acids in bold constitutes the intervening linker sequence

EXAMPLE 5
Bacterial Growth Conditions for Hybrid Polypeptide Production

The recombinant *V. cholerae* strains producing the hybrid protein polypeptides were cultured in a modified Syncase medium (Lebens et al., Biotechnology 11:1574-1578, 1993) with 100 μg/ml Ampicillin at 37°C with shaking. Samples were taken at 3, 6, 8, 13 and 24 h after inoculation and both the cells and the culture supernatant were analyzed in a GM1-ELISA (see Example 4 above and 6 below) with the mAb LT39-anti-CTB and F58/H3-anti-gp120. The major part of the produced hybrid proteins were found in the supernatant. The amount of CTB produced was calculated using a standard curve with purified recombinant CTB. The titers of F58/H3 binding were defined as the interpolated dilution giving an A_{450} of 0.4 above background.

Cultures of *E. coli* were grown in the same way using L-broth rather than syncase medium.

EXAMPLE 6
Screening for Expression of the Hybrid Proteins:

Characterization of the different internal hybrid proteins

The internal HIV::CTB, HBV::CTB and ST::CTB hybrid proteins were synthesized in the *V. cholerae* strain JS1569 (*ctxA*$^-$, *ctx*$_+$) which was transformed with the different plasmids by electroporation. The resulting recombinant bacterial strains were cultured at 37°C in the presence of 100 μg/ml Ampicillin. Hybrid protein was found in the culture medium which could be precipitated by acidification in the presence of hexametaphosphate (Lebens et al., *BioTechniques* 1993). Precipitates were redissolved and dialyzed extensively against PBS and then analyzed in SDS-PAGE and immunoblot.

Polyacrylamide Gel Analysis and Immunoblotting

1-3 μg of purified recombinant CTB and hybrid proteins were partially purified from culture supernatants by precipitation with sodium hexametaphosphate, as described by Lebens et al. (*Biotechniques*, 1993). Samples were prepared in Laemmli sample buffer with β-mercaptoethanol and were loaded both boiled and unboiled onto 15-17% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS). This separation provides visualization of bands corresponding to both the monomeric and pentameric forms of the proteins. The electrophoresis was performed at a constant voltage of 200 V for approximately 1 h. Gels were either stained with Coomassie Brilliant Blue or electrophoretically transferred to nitrocellulose membranes for immunoblot analyses. After blocking with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), the membranes were incubated with either the two CTB-specific mAb LT39 and CT6, reacting with pentameric and monomeric CTB respectively, mAb P4/D10 against gp120, mAb ST1:3 against ST$_1$ or mAb 5520 against pre-S(2). Horse-radish peroxidase labelled goat-anti-mouse IgG (Jackson) was used as secondary antibody and the membranes were developed with the chromogenic substrate 4-chloro-1-naphthol (Bio-Rad).

The CTB::HIV fusion protein migrated like native CTB in standard Coomassie-blue stained PAGE in both pentameric and monomeric forms. The inserted heterologous gp120 epitope in the hybrid protein was detected in standard immunoblots using mAb F58/H3, directed against the V3 loop of gp120. The gp120 epitope was recognized both when the hybrid protein was present in the assembled pentameric form of the protein and after dissociation into monomers. This also indicated that there was a significant retention of three-dimensional structure in the assembled pentamers.

The internal CTB::HIV hybrid protein showed an increasing titer with mAb F58/H3 proportional to the amount of protein produced and secreted. The peptide was resistant to proteolytic degradation when placed internally in CTB.

The additional HIV epitope - containing plasmids producing HIV::CTB hybrid proteins, pCB55-65gp309-318, pCB52-58gp309-318 and pCB55-64gp309-322, did not produce detectable levels of CTB hybrid protein. The reason for this is likely due to instability of the resulting proteins and degradation of the polypeptide chains. The structural differences between the proteins which were produced in detectable levels and the proteins which were not produced at detectable levels were minimal.

The ST::CTB hybrid proteins, encoded by the plasmids pCB55-64ST_{aaa} (strain 551) and pCB55-64ST_a (strain 557) were both produced in slightly higher amounts than the HIV::CTB and HBV::CTB chimeras, even in those proteins where the entire ST_a 19 aa fragment was inserted between amino acids 55 and 64 of CTB. Comparative expression levels were 15-30 μg/ml for pCB55-64ST_{aaa} (strain 551) and pCB55-64ST_a (strain 557) as compared with 5-15 μg/ml for the HBV::CTB and the HIV::CTB chimeras encoded by the plasmids pCB55-64gp309-318, pCB53-64gp309-318 and pCB53-64gp307-318. Thus, the amino acid composition of the foreign insert is also important. The ST sequences include several cysteines which may form internal disulfide bridges that potentially stabilize the structure of the inserted peptide and of the entire chimeric protein.

The HIV::CTB protein encoded by the plasmid pCB56gp309-318 (strain 439), having a straight insertion of the gp120 epitope without any deletions from CTB, was produced at a much higher level than the other proteins (up to 300 μg/ml after 24 hours). When analyzed in SDS-PAGE and immunoblot assays, the protein migrated as a sharp band of the size of pentameric CTB when analyzed unboiled. However, the main part of the protein fell apart when boiled in sample buffer containing SDS and β-mercaptoethanol. The HBV::CTB hybrid protein from strain 549, with a peptide from pre-S(2) inserted at the same position, behaved in exactly the same way. Resistance to proteolytic cleavage is probably dependent on the conformation and accessibility of the peptide in each individual hybrid protein.

It is worth noting that proteins can maintain a conformation which resembles CTB enough to migrate as CTB pentamers on a polyacrylamide gel, bind to GM1-gangliosides and be recognized by mAb LT39-anti-CTB, even after they have been cleaved at the position of the inserted peptide. Thus, further analysis of some clones may be necessary to ensure that they are not unduly susceptible to cleavage.

Analysis of the hybrid protein in GM1-ELISA Assays

Cholera toxin protein was detected using a GM1-ELISA that has been disclosed in the art (see Sanchez et al., *FEBS Lett.* 241:110-114, 1988 and Svennerholm et al., *J. Clin. Microbiol.* 24:585-590, 1986). Microtiter wells were coated overnight at room temperature with 0.3 nmol GM1-ganglioside (Sigma, St. Louis, Missouri) in 100 µl PBS and blocked with 0.1% BSA in PBS (PBS-BSA) for 30 min at 37°C. After three washings with PBS, samples were diluted in PBS-BSA and incubated for one hour. Recombinant CTB was also used at concentrations starting at 0.5 µg/ml. This and all subsequent incubations were performed at ambient temperature. After subsequent washings with 0.05% Tween-20 in PBS (PBS-T), either mAb LT39-anti-CTB, or foreign epitope specific antibody such as mAb F57/H3-anti-V3 loop of gp120, TB mAb P4/D10-anti-gp120, ST1:3-anti-STa or 5520-anti-pre-S(2), were added in PBS with 0.1% BSA and 0.05% Tween-20 (PBS-BSA-T) and incubated for one hour. Horse-radish peroxidase labelled goat-anti-mouse-IgG (Jackson Laboratories) in PBS-BSA-T was added as second antibody to the wells after washings with PBS-T, and after one hour the plates were washed again and then developed with the chromogenic substrate orthophenylenediamine (OPD) in citrate buffer with 0.012% H_2O_2. The absorbance was measured at 450 nm after 10-20 min. The amounts of each hybrid protein added in the first well of the microtiter plate was adjusted to 10 µg as estimated from Coomassie stained polyacrylamide gels with recombinant CTB as a standard and were also confirmed by the reaction with mAb LT39-anti-CTB. Titers were defined as the interpolated serum dilution giving a A_{450} of 0.2 above background.

Analysis of the internal gp120 epitope hybrid protein in a GM1-ELISA indicated that GM1 binding was retained together with affinity for the CTB pentamer-specific mAb, LT39. Under the same conditions, the CTB::HIV protein also reacted with the anti-gp120 mAb F58/H3, indicating that the gp120 epitope was exposed on the surface of the molecule. The ELISA assays for GM1 expression used the mAb LT39 and included a standard curve with recombinant CTB starting at 0.5 µg/ml, and reactivity with mAb P4/D10, which was defined as the interpolated serum dilution giving a A_{450} of 0.4 above background.

Antigenic properties of the gp120 epitope in the HIV::CTB proteins

The ten amino acid-peptide from gp120 (aa 309-318) yielded a relatively strong signal when placed N-terminally in CTB (see Example 3), or between aa 55 and 64, indicating that the epitope was surface exposed in these two proteins (from strain 504 and 407). When increasing the length of the inserted epitope to twelve aa (as in strain 586), the reaction of the epitope with antibody was weaker. Either the epitope was less well exposed on the surface or it adopted a conformation which the mAb did not recognize.

The HIV::CTB hybrid proteins were also analyzed in immunoblot with the same anti-gp120-mAb. When run in the unboiled pentameric form, the inserted gp120 epitope was only detected in the constructs with substitutions within amino acid positions 55-64. In all the other HIV::CTB proteins, the epitope could only be detected in the boiled monomeric form. This could imply that the epitope was exposed in the monomers form, but became more buried or distorted when pentamers were formed. At the same time,

the reaction in GM1-ELISA assays indicated that after binding to GM1, the pentameric forms of some of the proteins exposed the inserted HIV epitope enough to be recognized by the anti-gp120 monoclonal antibody. It is possible that the binding to GM1 may therefore facilitate the accessibility of the inserted HIV epitope on the pentamer surface.

The protein from strain 439, which is cleaved after it has been synthesized, was also analyzed in immunoblot with monoclonal P4/D10. The weak monomeric band of around 11 kD, which presumably is still uncleaved, reacts with the mAb, but the majority of the protein, which was degraded, showed no anti-gp120 reactivity.

Antigenic properties of the ST and pre-S(2) epitopes in the ST::CTB and HBV::CTB hybrid proteins

When analyzed in GM1-ELISA, the ST_a epitopes inserted between amino acids 55 and 64 in CTB strains 551 and 557 were detected with the ST-specific neutralizing mAb ST1:3 (see Svennerholm et al., *J. Clin. Microbiol.* 24: 585-590, 1986), and reacted more strongly than when these ST peptides were placed N- or C-terminally in CTB (see Sanchez et al., *Res. Microbiol.* 47:971-979, 1990). In immunoblot assays, the intrachain ST_a peptides were only detected in the monomeric protein form. This is consistent with what was seen with most of the HIV::CTB proteins. The same was true for the HBV::CTB protein, where the pre-S(2) sequence could not be detected with mAb 5520-anti-pre-S(2) (see Milich et al., *J. Immunol.* 137: 2703-2710, 1986) either in GM1-ELISA or in the unboiled pentameric form in immunoblot assays, but were readily observed in the boiled monomeric form.

These results indicated that when foreign antigen was inserted into internally in CTB within the 56-64 amino acid region, Ala-64 should be positioned C-terminally to the insert to get production of the protein. This observation is consistent with other scientists who have reported that Ala-64 is important for the stability of pentameric CTB. Likewise, the N-terminal position of Pro-53 was important to the insert since when deleted, as in pCB52-58gp309-318, the corresponding hybrid protein was not produced at detectable levels.

Based on ELISA assays to detect antibody binding to the toxin or to the foreign antigen, it was determined that the level of foreign epitope reactivity was somewhat less than the level of reactivity of the CTB epitopes. Since the intrachain fusion protein was resistant to proteolytic cleavage during production in *V. cholerae*, degradation of the foreign peptide epitope is probably not the explanation for the reduced immunogenicity of the foreign epitope in the hybrid protein as compared with level of CTB reactivity. It is more likely that the surface density of the inserted foreign epitope is low compared to the several different, strong, mainly conformational CTB epitopes. Thus, this invention also contemplates that multiple copies of the foreign antigen will by useful for promoting an increased immune response.

It is additionally contemplated that the length of the insert will also affect stability. Thus, those skilled in the art should also contemplate varying the length of the insert in those substitutions where stability is potentially a problem.

The ELISA screening methods in combination with polyacrylamide gel electrophoresis staining and immunoblot analysis serve to guide those skilled in the art to prepare appropriate combinations and to screen these combinations for expressed protein. Importantly, the results provided here indicated that CTB can be modified without loss of synthesis of the desired protein.

EXAMPLE 7
Immunization Protocol for Optimizing Serum Antibody Levels

Female C57B1/6 mice were immunized intraperitoneally (i.p.) with three doses of 10 μg of the CTB::HIV hybrid protein partially purified from JS1569 supernatant. The first dose was given with Freund's complete adjuvant (DIFCO Laboratories, Detroit, Michigan) and the subsequent doses with Freund's incomplete adjuvant. As negative controls, mice were immunized using the same scheme with a CTB hybrid protein with an irrelevant foreign epitope or with Freund's adjuvant alone. Serum samples were collected before the first dose and seven days after the second and third dose. Sera were analyzed in ELISA, using either CTB (bound to GM1 in GM1-coated microwells using the methods of Example 6) or recombinant gp120 (Bolmstedt et al., *J. Gen. Virol.* 73:3099-3105, 1992) as antigen.

A strong serum antibody response to the CTB moiety was detected in all mice immunized with either of two hybrid proteins. There was also a significant serum antibody response against the HIV moiety in the majority of the mice tested. The mice receiving only Freund's adjuvant gave no antibody responses against either CTB or gp120.

EXAMPLE 8
Immunization Protocol for Producing Vaginal Immune response

Female mice were given 10 mg of progesterone subcutaneously 10 and 3 days before the first immunization and were then treated once a week with progesterone. Groups of mice were immunized either four times intravaginally (at 1-2 week intervals between each dose) or with three intraperitoneal doses followed by an intravaginal dose. Each intravaginal immunization dose consisted of ca. 0.5 mg CTB-peptide conjugate (see Example 1). Each dose was estimated to contain approximately 0.4 mg CTB and 0.1 mg A8-VDIV peptide and additionally included 5 mcg cholera toxin as extra adjuvant and each intraperitoneal dose contained one third this amount.

EXAMPLE 9
Immunization to produce
Mucosal Antibody

The CTB fusion proteins are isolated from bacterial culture supernatants using the expression system described by Lebens, et al. (*BioTechnology, supra*). The fusion proteins are isolated by GM1 affinity purification as disclosed by Tayot, et al. (*Europ. J. Biochem.* 113: 249-258) and dialyzed against PBS. The specific protein concentrations of the samples used for immunization are determined by ELISA using a CTB standard.

In an experimental model, male monkeys are immunized with 40-250 μg of CTB equivalents of protein in each immunization. In total, 3-5 injections were give every 3-4 weeks, the first three with the antigen suspended in complete Freund adjuvant and subsequent immunizations in incomplete adjuvant. Sera prepared from the bleedings taken before the start of the immunizations and those taken 2 weeks after the third or fourth vaccination were assayed for specific antibodies against CTB and the foreign antigen by ELISA. In addition, the ability of the sera to neutralize the pathogen was assayed in the infant mouse test. Serum from immunized monkeys in a dilution of 1/5 was mixed with an equal volume of pathogen and introduced into the mouths of infant mice. Mice were monitored for the presence of the disease over time.

For human immunization it is contemplated that the chimeric protein will be administered once or on repeated occasions by the oral, rectal, vaginal or nasal routes using between 0.1-2 mg of construct for single immunizations and between 0.01-0.2 mg for repeated immunizations. The protein may be given either in a liquid form or dispersed in an inert gel with estimated volumes of inoculum of between 0.1-1 ml for injection, 0.5 - 2 ml for nasal and between 3-10 ml for rectal or vaginal immunization. It is contemplated that the oral immunization is likely to be given together in a pharmaceutical acceptable buffer containing about 25-200 ml liquid containing about 2 grams of sodium bicarbonate or equivalent acid buffering agent.

EXAMPLE 10
Experimental Screening for IgA and IgG Mucosal Antibody

Serum, mesenteric lymphnodes, spleen, cervix, vagina, small intestine, colon and rectum were collected from mice sacrificed (and perfused with heparin containing buffer) one week after the last immunization dose with the preparation of Example 1 using the immunization protocol of Example 8. The organs were frozen and then extracted with 2% (W/V) of saponin using the PERFEXT method (Quiding, et al. *J. Clin. Invest.* 88(1):143-148, 1991). The saponin extracts were tested for IgA and IgG anti-CTB and anti-VDIV antibodies by ELISA. ELISA plates were coated with GM1 (0.3 nmol/ml) or A8-VDIV peptide (1 μg/ml) respectively.

In this example, immunized mice were of the C57/B1 strain and were 8-10 weeks old at the onset of immunizations. The immunization protocol was 1 mg medroxyprogesterone acetate (Depoprovera; UpJohn Company, Kalamazoo, MI) subcutaneously ten and three days before the first immunization and then once weekly through the course of the immunization period. Immunizations were given by the indicated routes with an interval of two weeks between the first and second dose and then one week between the following doses.

TABLE 2. IMMUNE RESPONSES IN THE FEMALE GENITAL TRACT AND SERUM OF MICE IMMUNIZED BY VARIOUS ROUTES WITH A CHEMICALLY PREPARED CONJUGATE BETWEEN CHOLERA TOXIN B SUBUNIT AND THE A8-VDIV CO-LINEAR PEPTIDE DERIVED FROM THE MAJOR OUTER MEMBRANE PROTEIN OF CHLAMYDIA TRACHOMATIS

	ELISA antibody titer to A8-VDIV							
Immunization	Vagina		Cervix uteri		Fallopian tubes		Serum	
	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
None	<.5	<.5	<.5	<.5	<.5	<.5	<.5	<.5
Intravaginal x 4	290	.5	920	.5	50	.5	3	43
Oral x 4	<.5	<.5	<.5	<.5	22	<.5	<.5	3
Intraperitoneal x 3 + intravaginal x 1	14	99	40	130	38	nd	2	3500
Oral x 4 + intravaginal x 1	2	<.5	2	<.5	<.5	<.5	<.5	<.5

This table indicates that with repeated intravaginal immunization or a combination of repeated intraperitoneal priming followed by a vaginal booster immunization, the CTB-A8-VDIV conjugate could induce substantial specific genital mucosal IgA antibody formation to the *Chlamydia trachomatis* A8-VDIV antigen in test samples comprising the vaginal, cervical and Fallopian tube mucosae. After multiple intraperitoneal immunizations, the IgA response was also associated with a substantial IgG response in both the genital tract tissues and in serum.

Immunogenicity of the foreign sequences in the CTB hybrid proteins

To determine the ability of the internally inserted peptides to induce immune responses, groups of mice of strain C57B1/6 were immunized intraperitoneally (i.p.) with either the internal HIV::CTB hybrid proteins from strain 407, 460 or 586, the N-terminal HIV::CTB hybrid protein from strain 504, or the two ST::CTB chimeric proteins, with Freund's adjuvant. All mice responded with high titered serum anti-CTB-IgG-responses. As noted above, the ten amino acid HIV epitope induced serum IgG-responses against gp120 when placed between amino acids 55 and 64 of CTB. A weak response against gp120 was also induced by the gp120 moiety in the mice immunized with the internal HIV::CTB hybrids from strain 460 (ten gp120 amino acids between 53 and 64) and 586 (twelve gp120 amino acids between 53 and 64) and by the N-

terminally placed gp120-epitope in the protein from strain 504. In fact, all these proteins showed immunogenic properties similar to the previously described HIV::CTB protein.

The ST decapeptide in the ST::CTB hybrid protein from strain 551 induced anti-ST-serum antibodies of relatively high titer in some of the animals, even though the immunological reactivity of the inserted peptide was modest *in vitro*. The ST::CTB protein from strain 557, which represented the entire ST, peptide, was also immunogenic in terms of the ST moiety, but induced lower titers of antibody than the decapeptide in the protein from strain 551.

The HBV::CTB chimeric protein from strain 395 was given either i.p. or perorally (p.o.) with cholera toxin (CT) as adjuvant to either BALB/c or C57B1/6 mice. High serum-anti-CTB titers where obtained in both p.o. and i.p. immunized mice, whereas the most significant levels of anti-pre-S(2)-ab were induced with i.p. immunizations using CT as adjuvant. Table 3 illustrates the various immunization protocols and their results.

Table 3. Serum IgG titers after immunization with the HBV::CTB hybrid protein.

Mouse strain	CT[a]	Route[b]	anti-CTB[c]		anti-pre-S(2)[d]	
			2 doses	3 doses	2 doses	3 doses
C57BL/6 (H-2[b])	-	p.o.	53 000[e]	23 400	0	0
BALB/c (H-2[d])	-	p.o.	0	2 400	0	0
C57BL/6	+	p.o.	102 400	256 400	0	0
BALB/c	+	p.o.	4 000	38 800	0	0
C57BL/6	-	i.p.	170 000	546 000	0	1 060
BALB/c	-	i.p.	32 000	153 600	0	0
C57BL/6	+	i.p.	10^6	682 000	8 500	3 300
BALB/c	+	i.p.	10^6	461 000	6 400	3 600

a) Mice were immunized with or without 5 μg (p.o.) or 1.5 μg (i.p.) cholera toxin (CT) as adjuvant.

b) The animals were given does of 30 μg (p.o.) or 7.5 μg (i.p.) of the HBV::CTB hybrid protein from strain 395 on day 0, 14 and 24.

c) Recombinant LTB bound to GM1 was used as antigen for anti-CTB-IgG determinations.

d) A synthetic peptide containing aa residues 133-153 from pre-S(2) (Schödel et al., 1990) was used as antigen for anti-preS(2)-IgG determinations.

e) Sera were collected on day 7 after does 2 and 3 and sera from mice from the same group (2 or 3 mice per group were pooled before analyses. Mean reciprocal serum dilutions yielding an $A_{392}>3$ x the A_{492} of preimmune sera are indicated as titers.

While particular embodiments of the invention have been described in detail, it will be apparent to those skilled in the art that these embodiments are exemplary rather than limiting, and the true scope of the invention is that defined in the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Holmgren, Jan
Lebens, Michael

(ii) TITLE OF THE INVENTION: IMMUNOGENS FOR STIMULATING MUCOSAL IMMUNITY

(iii) NUMBER OF SEQUENCES: 51

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(B) STREET: 620 Newport Center Drive 16th Floor
(C) CITY: Newport Beach
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 92660

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kaiser, AnneMarie
(B) REGISTRATION NUMBER: 37,649
(C) REFERENCE/DOCKET NUMBER: HOLMG.001VPC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 619-235-8550
(B) TELEFAX: 619-235-0176
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Phe Asp Val Thr Thr Leu Asn Pro Thr Ile Ala Gly Ala Gly Asp Val
1 5 10 15
Lys

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Leu Asn Ile Trp Asp Arg Phe Asp Val Phe Cys Thr Leu Gly Ala
1 5 10 15
Thr Thr Gly Tyr Leu Lys Gly Asn Ser Phe Asp Val Thr Thr Leu Asn
20 25 30
Pro Thr Ile Ala Gly Ala Gly Asp Val Lys
35 40

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGGCGCGCT TTCGTTGCGA TCGAAAGGAT GAAGGATACC CTGAGGATTG C 51

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGGCGCGCC CCGGACCACG CTGGATACTA CCTGGTACCT CTACTTGAAA AGTTGC 56

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 125 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 33...125
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
AATTCGATTC TGTTAAACAA AGGGAGCATT AT ATG GTA AAG ATA ATA TTT GTG     53
                                    Met Val Lys Ile Ile Phe Val
                                     1               5

TTT TTC TTA TCA TCA TTT TCA TAT GCA AAT GAT GAT AAG TTA GGA GCT    101
Phe Phe Leu Ser Ser Phe Ser Tyr Ala Asn Asp Asp Lys Leu Gly Ala
        10                  15                  20

CCT GAT TCT AGA GCG ATG AGT AAT                                   125
Pro Asp Ser Arg Ala Met Ser Asn
    25                  30
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Ala Leu Asn Ile Trp Asp Arg Phe Asp Val Phe Cys Thr Leu Gly Ala
 1               5                  10                  15
Thr Thr Gly Tyr Leu Lys Gly Asn Ser
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCGGCTAGCG CAGCT 15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCGCTAGCCG GAGCT 15

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCTATTCAGC GTGGTCCGGG GCGCGCTTTT GTTGCTCCTC AAAAT 45

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATTTTGACCA GCAACAAAAG CGCGCCCCGG ACCACGCTGA ATAGGAGCT 49

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCTCAAATTC AGCGTGGTCC GGGGCGCGCT TTTGTTAAC 39

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTTAACTTTA GCCGCGCCCC GGACCACGCT GAATTTGAGG AGCT 44

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Ala Leu Asn Ile Trp Asp Arg Phe Asp Val Phe Cys Thr Leu Gly Ala
 1               5                  10                  15
Thr Thr Gly Tyr Leu Lys Gly Asn Ser Phe Asp Val Thr Thr Leu Asn
            20                  25                  30
Pro Thr Ile Ala Gly Ala Gly Asp Val Lys
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Cys Ala Leu Asn Ile Trp Asp Arg Phe Asp Val Phe Cys Thr Leu Gly
 1               5                  10                  15
Ala Thr Thr Gly Tyr Leu Lys Gly Asn Ser Phe Asp Val Thr Thr Leu
            20                  25                  30
Asn Pro Thr Ile Ala Gly Ala Gly Asp Val Lys
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Ala Leu Asn Ile Trp Asp Arg Phe Asp Val Phe Cys Thr Leu Gly Ala
 1               5                  10                  15
Thr Thr Gly Tyr Leu Lys Gly Asn Ser Phe Asp Val Thr Thr Leu Asn
            20                  25                  30
Pro Thr Ile Ala Gly Ala Gly Asp Val Lys Cys
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
GGGGGGGATC CCCTCGCGCG TTTCGGTGAT GAC                              33
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGGGGAGATC TCTGAAATGA GCTGTTGACA ATTATC 36

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGGGGAGATC TGCCAGAACC GTTATGATGT CGG 33

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGGGGGGATC CCGAACGCCA GCAAAGACGT A 31

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATTCAGCGTG GTCCGGGTCG TGCTTTTG 28

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CCGGGAAAAC GACGACCCGG ACCACGCTGA ATAGCT 36

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCGTAGCGGT ACCACCACTC TGAACCCAAC TATTGCTGGA GCTGGCTGGC CAGCACG 57

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCGTGCTGGC CAGCCAGCTC CAGCAATAGT TGGGTTCAGA GTGGTGGTAC CGCTACGC 58

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCATTTGATA TTACCACTTT AAATCCAACA ATTGCTGGTG CTGGTGATGT TAAACCCGGG 60
T 61

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTAGACCCGG GTTTAACATC ACCAGCACCA GCACCAGCAA TTGTTGGATT TAAAGTGGTA 60
ATATCAAATG GAGCT 75

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 76 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCCGCTTTAA ATATTTGGGA TCGTTTTGAT GTTTTTTGTA CATTAGGTGC TACCACTGGT 60
TATAAAGGTA ATAGTT 76

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 84 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTAGAACTAT TACCAAAATA ACCAGTGGTA GCACCTAATG TACATTTAAC ATCAAAACGA 60
TCCCAAATAT TTAAAGCGGG AGCT 84

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Asp Pro Arg Val Arg Gly Leu Tyr Phe Pro Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Ile Gln Arg Gly Pro Gly Arg Ala Phe Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Cys Ala Glu Leu Cys Cys Asn Pro Ala Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```
Asn Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr
 1               5                  10                  15
Gly Cys Tyr
```

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```
GGGGCGCGCT TTCGTTGCGA TCGAAAGGAT GAAGGATACC CTGAGGATTG C          51
```

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
GGGGCGCGCC CCGGACCACG CTGGATACTA CCTGGTACCT CTACTTGAAA AGTTGCACCA  60
TTCTTAAAAG                                                        70
```

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GGGGCGCGCT TTCGTTCATA TAGATTCACA AAAAAAAGCG AT 42

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GGGGCGCGCC CCGGACCACG CTGGATTTGA CTACCTGGTA CTTCT 45

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GGGGCGCGCT TTCGTTATAG ATTCACAAAA AAAAGCGAT 39

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GGGGCGCGCC CCGGACCACG CTGGATTACT TCTACTTGAA AAGTTGCA 48

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CAATCCAGCG TGGTCCGGGG 20

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CGCGCCCCGG ACCACGCTGG ATTGGTAC 28

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CAATCCGGAT CCAGCGTGGT CCGGGG 26

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CGCGCCCCGG ACCACGCTGG ATCCGGATTG GTAC 34

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

CTTTTCCTAT TGTAACGAAA GCGCGCCCCG GACCACGCTG GATCAAAAAT GCA 53

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

TTTTTGATCC AGCGTGGTCC GGGGCGCGCT TTCGTTACAA TAGGAAAA 48

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CAGGTAGTTG CGCTGAATTG TGTTGTAATC CTGCATGCG 39

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CGCATGCAGG ATTACAACAC AATTCAGCGC AACTACCTGG TAC 43

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CAGGTAGTAA TAGCAGCAAT TACTGCTGTG AATTGTGTTG TAATCCTGCA TGCACTGGAT 60
GTTACG 66

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CGTAACATCC AGTGCATGCA GGATTACAAC ACAATTCACA GCAGTAATTG CTGCTATTAC 60
TACCTGGTAC 70

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GGGCCTCGAG TCCGAGGCCT ATACTTTCCG GCGATTGAAA GGATGAAGGA TACCCTG 57

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GGGCTCGAGG GTCACTACCT GGTACCTCTA CTTGAAAAGT TG 42

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GGGCTCGAGG ATCTTGACTA CCTGGTACTT CTACTTGAAA AG 42

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

GGGCTCGAGT TCGTGGTCTG TACTTCCCGG CTCATATAGA TTCACAAAAA AAAGCGATTG 60
AAAG 64

WHAT IS CLAIMED IS:

1. A mucosal binding composition, comprising a mucosal binding polypeptide linked to at least one antigen of a non-viral pathogen, wherein said pathogen causes a sexually transmitted disease.

2. The composition of Claim 1, wherein said mucosal binding polypeptide is the binding subunit of cholera toxin.

3. The composition of Claim 2, wherein said composition additionally comprises the A subunit of cholera toxin or a portion thereof.

4. The composition of Claim 1, wherein the antigen is a chlamydia antigen.

5. The composition of Claim 4, wherein the chlamydia antigen is linked to the amino terminus of the binding subunit of cholera toxin.

6. The composition of Claim 4, wherein the chlamydia antigen is linked to an internal portion of the binding subunit of cholera toxin.

7. The composition of Claim 4, wherein said mucosal binding polypeptide is the binding subunit of cholera toxin and said antigen comprises a B-cell stimulating antigen from the major outer membrane protein of chlamydia.

8. The composition of Claim 4, wherein said B-cell stimulating antigen is from the VDIV region of the major outer membrane protein of chlamydia.

9. The composition of Claim 8, wherein said antigen further comprises a T-helper cell stimulating antigen from the major outer membrane protein of chlamydia.

10. The composition of Claim 9, wherein said T-helper cell stimulating antigen is from the A8 region.

11. The composition of Claim 1, wherein said antigen is chemically linked to said binding polypeptide.

12. The composition of Claim 1, wherein said antigen is linked to said binding polypeptide as a genetic fusion protein.

13. A method for generating a mucosal immune response against a non-viral sexually transmitted disease, comprising contacting the mucosa of a mammalian host with the composition of Claim 1.

14. The method of Claim 13, wherein the composition is the composition of Claim 2.

15. The method of Claim 13, wherein the composition is the composition of Claim 6.

16. A recombinant polynucleotide, comprising a first region encoding a mucosal binding polypeptide and a second region encoding an antigen of a non-viral pathogen, wherein said pathogen causes a sexually transmitted disease.

17. The polynucleotide of Claim 16, wherein said mucosal binding polypeptide is the binding subunit of cholera toxin and said pathogen is chlamydia.

18. The polynucleotide of Claim 17, wherein said antigen is a T-cell helper cell stimulating antigen from the major outer membrane protein of chlamydia.

19. The polynucleotide of Claim 18, wherein said T-cell helper stimulating antigen is from the A8 region.

20. The polynucleotide of Claim 19, wherein said antigen further comprises a B-cell stimulating antigen from the major outer membrane protein of chlamydia.

21. The polynucleotide of Claim 20, wherein said B-cell antigen is from the VDIV region of the major outer membrane protein of chlamydia.

22. A method for vaccinating against chlamydia infection, comprising administering to the mucosa of a mammalian host an effective amount of the binding subunit of cholera toxin linked to both a B-cell epitope and a T-cell epitope of the major outer membrane protein of chlamydia.

23. The method of Claim 22, wherein said administration is vaginal.

24. The method of Claim 22, wherein said administration is rectal.

25. The method of Claim 22, wherein said administration is oral.

26. A mucosal binding composition comprising a mucosal binding polypeptide linked to at least one antigen of a viral pathogen, wherein said pathogen causes a sexually transmitted disease.

27. The composition of Claim 26, wherein said mucosal binding polypeptide further comprises the binding subunit of cholera toxin.

28. The composition of Claim 27, wherein said antigen is a HIV gp120 antigen.

29. The composition of Claim 28, wherein said antigen is the peptide corresponding to SEQ ID NO: 29.

30. The composition of Claim 27, wherein said antigen is a Hepatitis B Virus antigen.

31. The composition of Claim 30, wherein said antigen is from the Hepatitis B virus pre-S(2) protein.

32. The composition of Claim 31, wherein said antigen is the peptide fragment corresponding to SEQ ID NO: 28.

33. A mucosal binding composition comprising a mucosal binding polypeptide linked to at least one antigen from an enterotoxigenic *E. coli*.

34. The composition of Claim 33, wherein said antigen is from the ST_1 protein of enterotoxigenic *E. coli*.

35. The composition of Claim 34, wherein said antigen is the peptide fragment corresponding to SEQ ID NO: 30.

36. The composition of Claim 34, wherein said antigen is the peptide fragment corresponding to SEQ ID NO: 31.

37. A purified recombinant polynucleotide comprising nucleic acid encoding a mucosal binding protein operably linked to a B-cell stimulating antigen, wherein said antigen is a peptide obtained from a pathogen capable of infecting a mammal through the mucosal membranes of that mammal.

38. The polynucleotide of Claim 37, wherein said nucleic acid encoding a mucosal binding protein encodes the binding subunit of cholera toxin.

39. The polynucleotide of Claim 38, wherein said nucleic acid further comprises nucleic acid encoding the CTA(2) subunit of cholera toxin.

40. The polynucleotide of Claim 39, wherein the nucleic acid encoding the B-cell stimulating antigen is positioned 5' to the nucleic acid encoding the CTA(2) subunit.

41. The polynucleotide of Claim 40, wherein the nucleic acid encoding the B-cell stimulating antigen encodes a peptide which includes the amino acid sequence LNPTIAG.

42. The polynucleotide of Claim 40, wherein the nucleic acid encoding the B-cell stimulating antigen encodes a peptide from HIV gp 120.

43. The polynucleotide of Claim 38, wherein the nucleic acid encoding the B-cell stimulating antigen is positioned in-frame within the coding region of the nucleic acid encoding said mucosal binding protein.

44. The polynucleotide of Claim 43, wherein said nucleic acid encoding the B-cell stimulating antigen encodes a peptide which includes the amino acid sequence LNPTIAG.

45. The polynucleotide of Claim 43, wherein said nucleic acid encoding the B-cell stimulating antigen encodes a peptide from HIV gp120.

46. The polynucleotide of Claim 45, wherein said nucleic acid encoding a peptide from HIV gp120, encodes the peptide IQRGPGRAFV.

47. The polynucleotide of Claim 43, wherein said nucleic acid encoding the B-cell stimulating antigen is from the Hepatitis B virus pre-S(2) protein.

48. The polynucleotide of Claim 47, wherein said nucleic acid encoding the B-cell stimulating antigen encodes peptide having the amino acid sequence of SEQ ID NO: 28.

49. The polynucleotide of Claim 43, wherein said nucleic acid encoding the B-cell stimulating antigen is from the ST_a protein of enterotoxigenic *E. coli*.

50. The polynucleotide of Claim 49, wherein said nucleic acid encoding the B-cell stimulating antigen encodes peptide having the amino acid sequence of SEQ ID NO: 30.

51. The polynucleotide of Claim 49, wherein said nucleic acid encoding the B-cell stimulating antigen encodes peptide having the amino acid sequence of SEQ ID NO: 31.

52. The polynucleotide of Claim 43, wherein the nucleic acid encoding the B-cell stimulating antigen is between 21 and 150 bases in length.

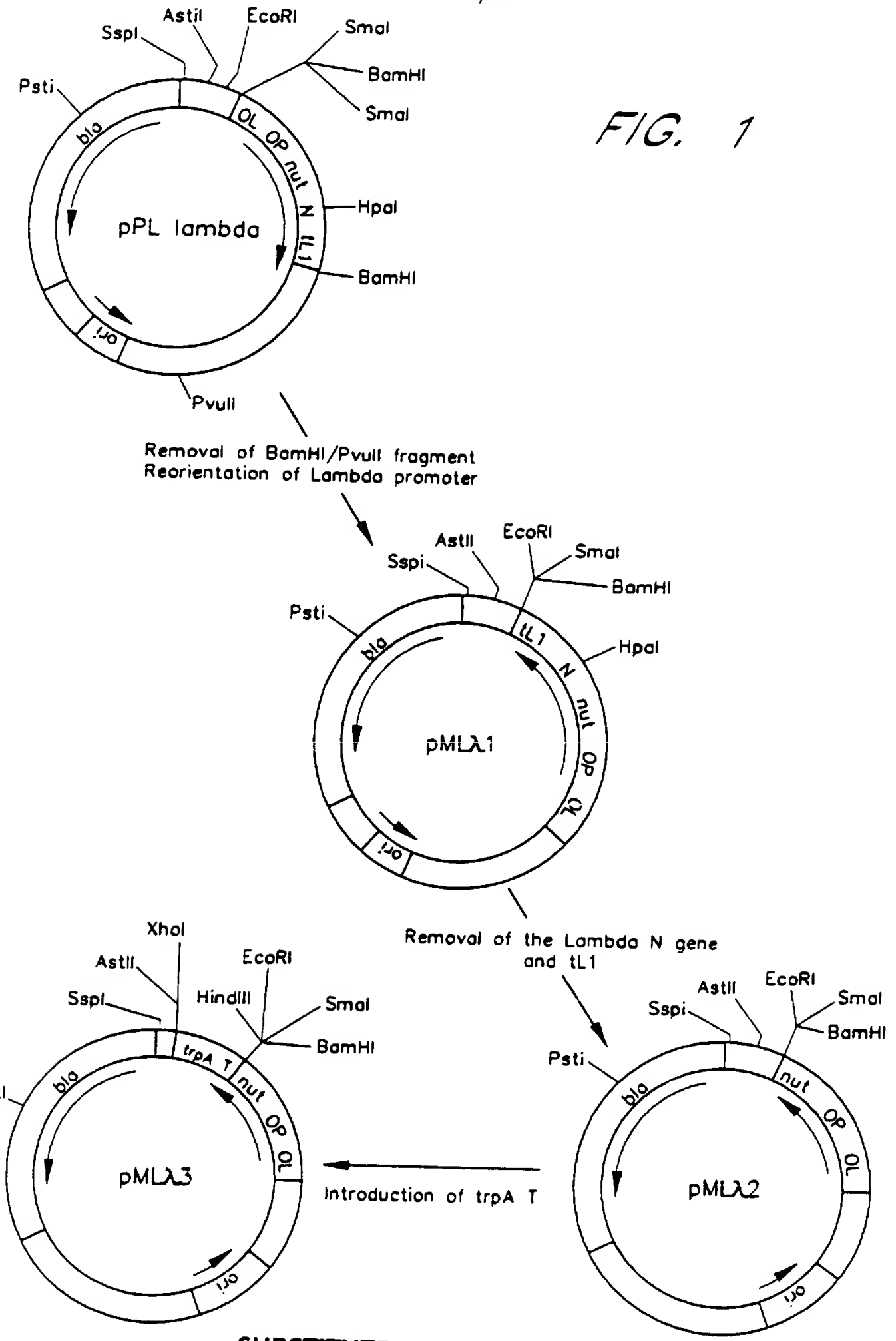
AstII
EcoRI
SspI
SmaI
BamHI
PstI
SmaI
bla
OL OP nut N tL1
pPL lambda
HpaI
BamHI
ori
PvuII
Removal of BamHI/PvuII fragment
Reorientation of Lambda promoter
AstII
EcoRI
SspI
SmaI
BamHI
PstI
bla
tL1 N nut OP OL
pMLλ1
HpaI
ori
Removal of the Lambda N gene
and tL1
XhoI
AstII
EcoRI
SspI
HindIII
SmaI
BamHI
PstI
bla
trpA T nut OP OL
pMLλ3
ori
Introduction of trpA T
AstII
EcoRI
SspI
SmaI
BamHI
PstI
bla
nut OP OL
pMLλ2
ori

FIG. 1

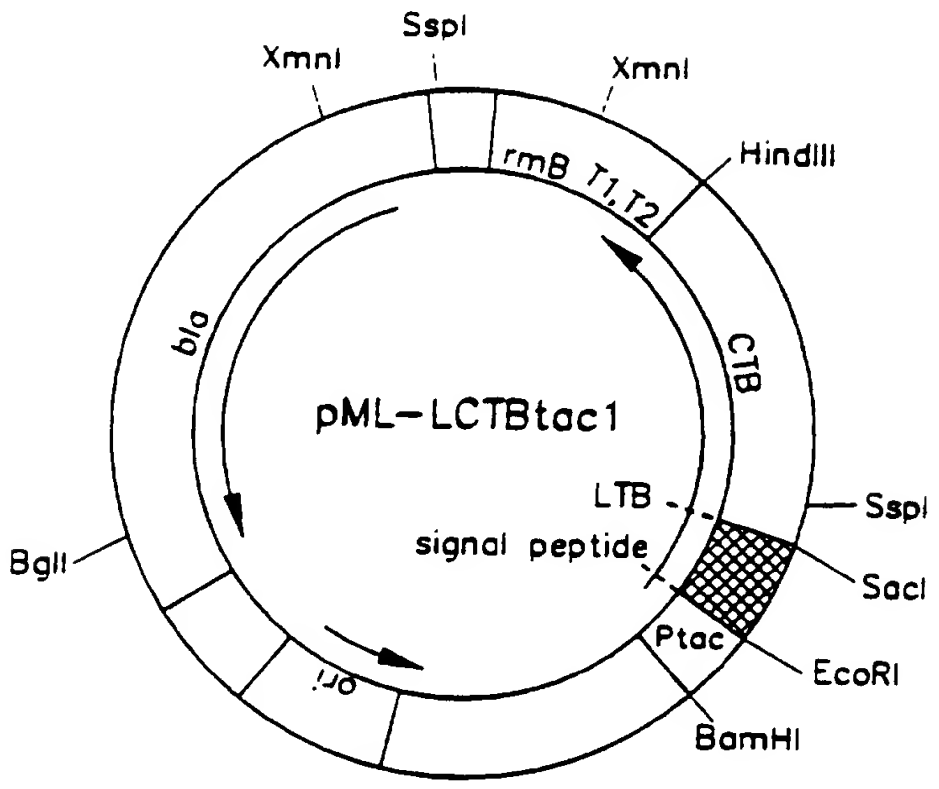
SspI
XmnI
XmnI
HindIII
rmB T1,T2
CTB
LTB
signal peptide
SspI
SacI
Ptac
EcoRI
BamHI
ori
BglI
bla
pML-LCTBtac1

FIG. 2a

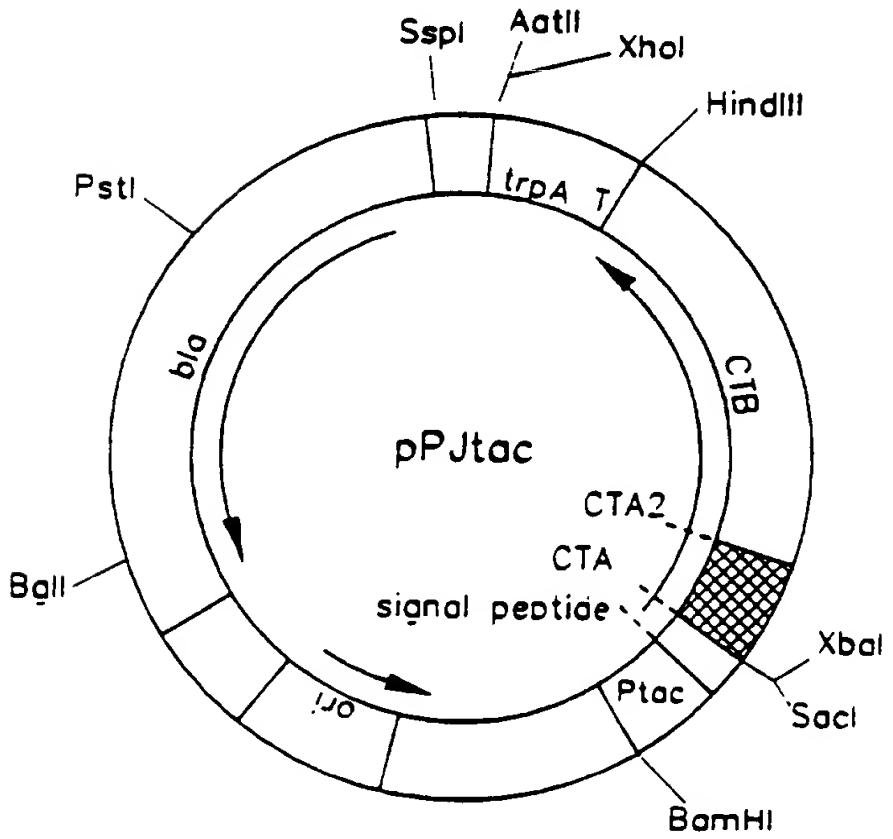
SspI
AatII
XhoI
HindIII
trpA T
CTB
CTA2
CTA
signal peptide
XbaI
SacI
Ptac
BamHI
ori
BglI
bla
PstI
pPJtac

FIG. 2b

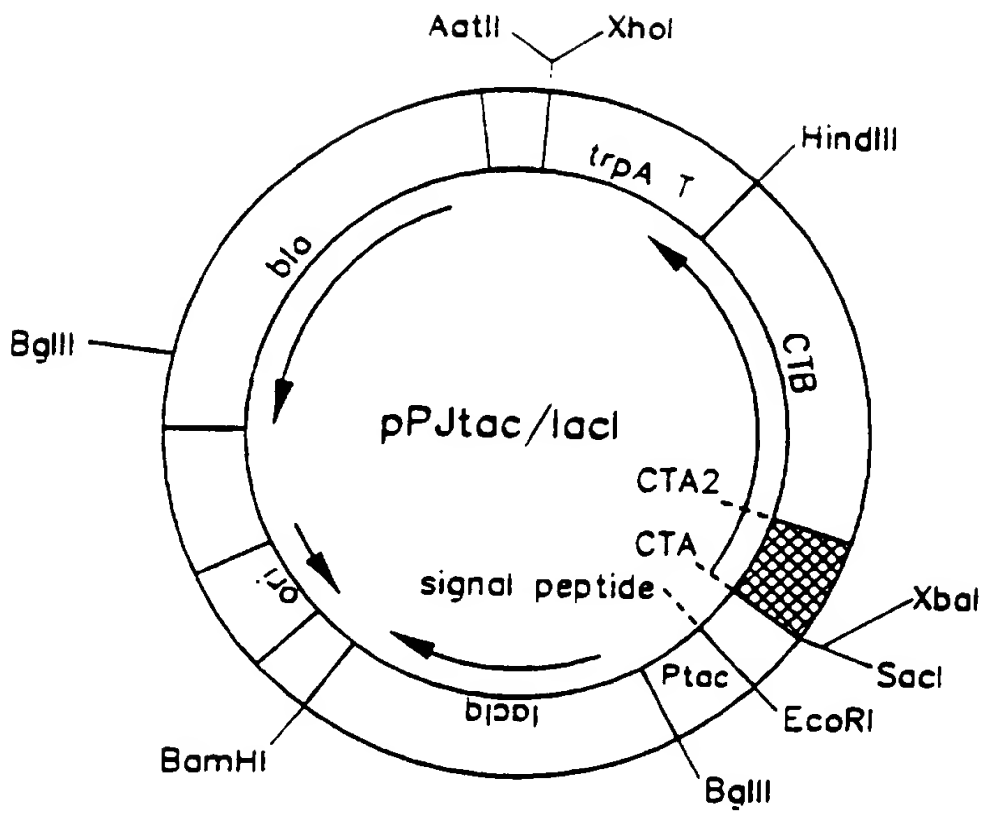
AatII
XhoI
HindIII
trpA T
bla
BglII
CTB
pPJtac/lacI
CTA2
CTA
signal peptide
ori
XbaI
SacI
Ptac
EcoRI
lacIq
BamHI
BglII

FIG. 2c

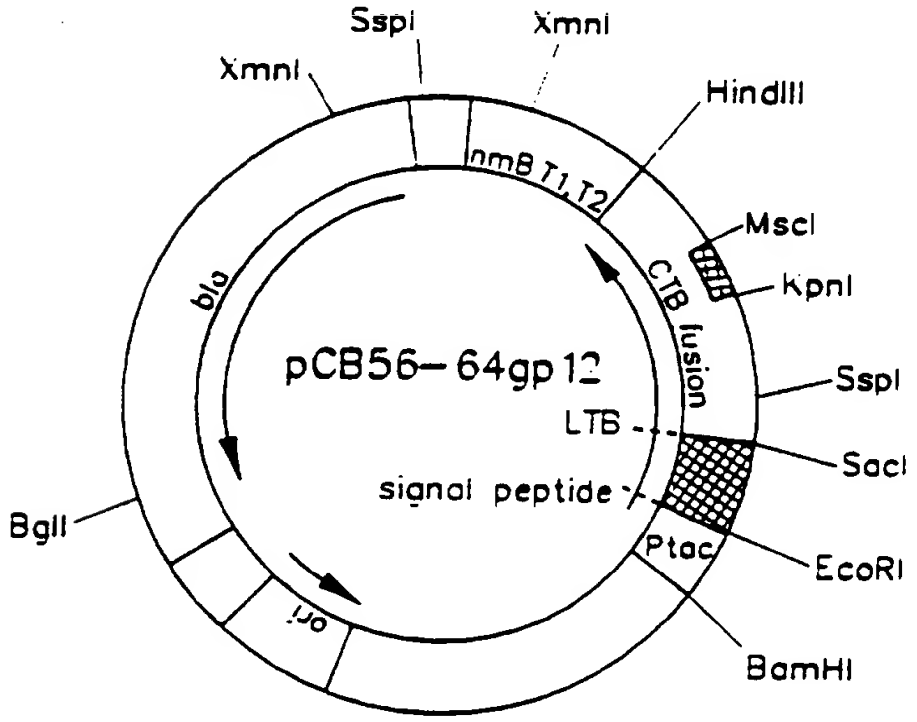
SspI
XmnI
XmnI
HindIII
nmB T1,T2
MscI
KpnI
bla
CTB fusion
pCB56–64gp12
SspI
LTB
SacI
signal peptide
BglI
Ptac
EcoRI
ori
BamHI

FIG. 2d

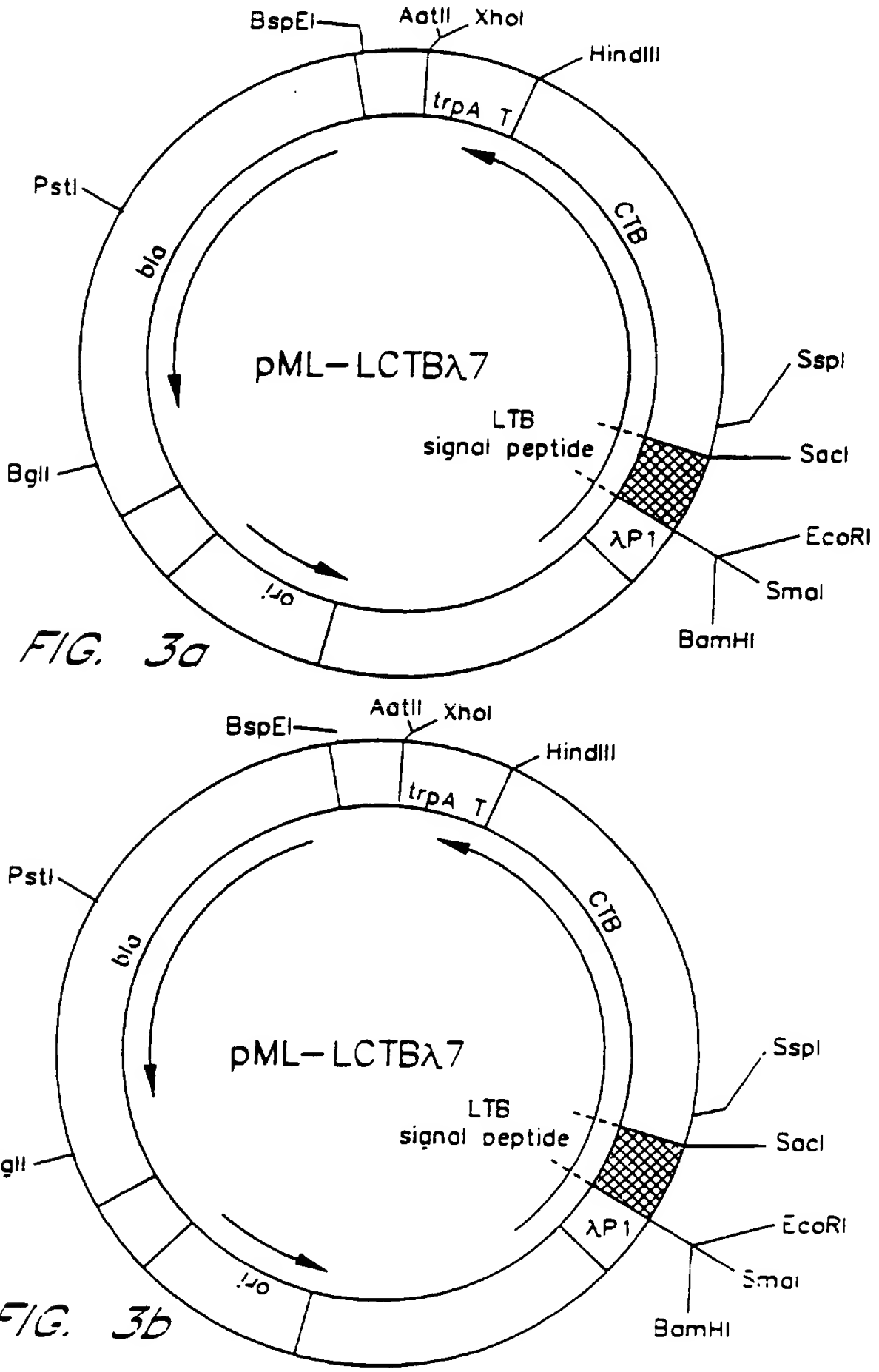
BspEI
AatII
XhoI
HindIII
trpA
T
PstI
blo
CTB
pML–LCTBλ7
SspI
LTB
signal peptide
SacI
BglI
λP1
EcoRI
SmaI
ori
BamHI

FIG. 3a

FIG. 3b

Oligonucleotide sequence used to reintroduce the clxA signal peptide sequence to CTA2

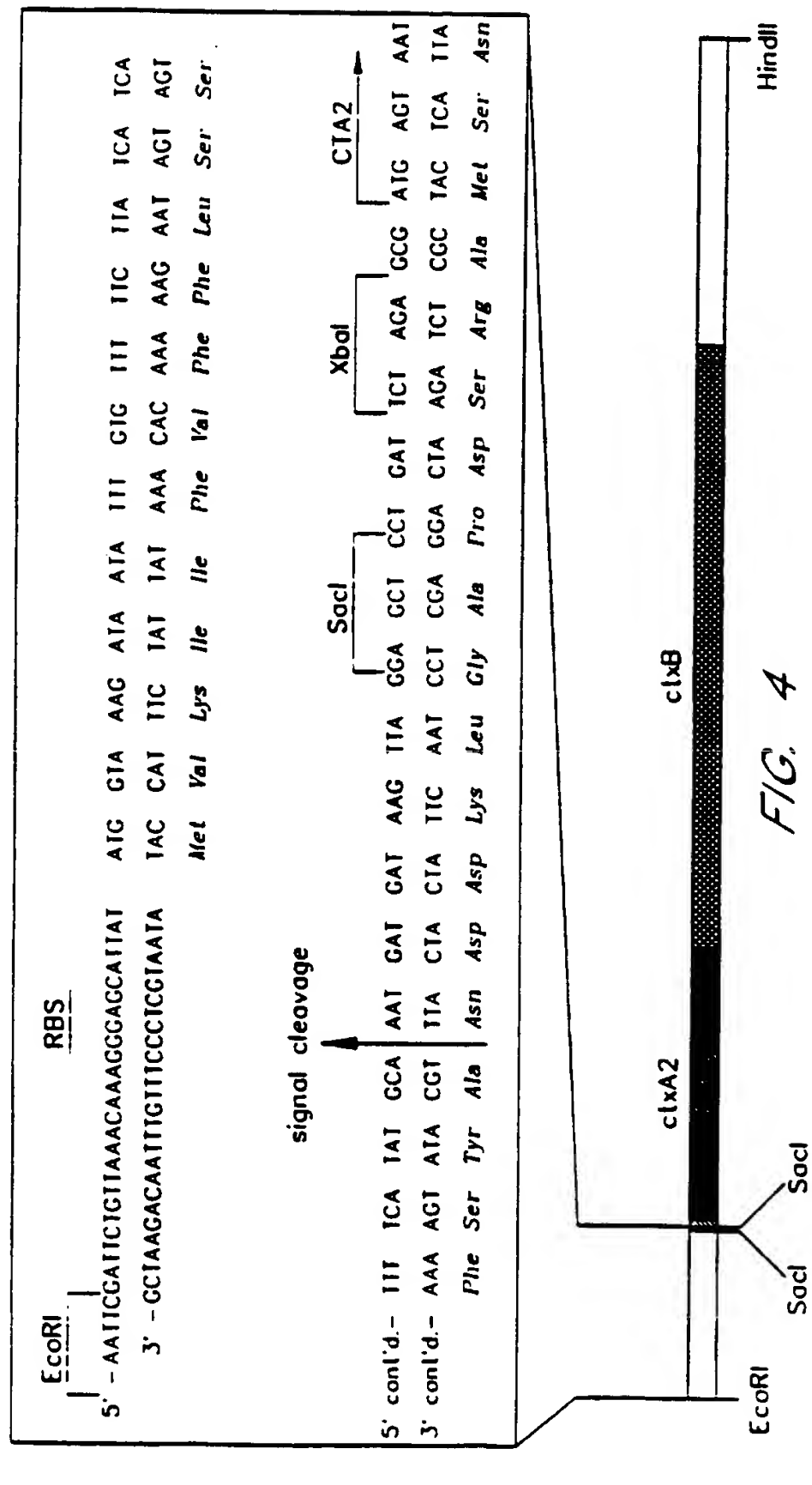

FIG. 4

Chlamydia B-cell and T-cell epitope sequences for insertion into cholera toxin gene fusions.

VD IV (B-cell epitope) : Uppersequence 5'-3"

SacI																	SmaI	XbaI	
	CCA	TTT	GAT	ATT	ACC	ACT	TTA	AAT	CCA	ACA	ATT	GCT	GGT	GCT	GGT	GAT	GTT	AAA	CCC GGG T
TCGAGGT		AAA	CTA	TAA	TGG	TGA	AAT	TTA	GGT	TGT	TAA	CGA	CCA	CGA	CCA	CTA	CAA	TTT	GGG CCC AGATC
	Pro	*Phe*	*Asp*	*Ile*	*Thr*	*Thr*	*Leu*	*Asn*	*Pro*	*Thr*	*Ile*	*Ala*	*Gly*	*Ala*	*Gly*	*Asp*	*Val*	*Lys*	*Pro* *Gly*

A 8 (T-cell epitope): Upper sequence 5'-3"

SacI																					
	CCC	GCT	TTA	AAT	ATT	TGG	GAT	CGT	TTT	GAT	GTT	TTT	TGT	ACA	TTA	GGT	GCT	ACC	ACT	GGT	TAT
TCGAGGG		CGA	AAT	TTA	TAA	ACC	CTA	GCA	AAA	CTA	CAA	TTT	ACA	TGT	AAT	CCA	CGA	TGG	TGA	CCA	ATA
	Pro	*Ala*	*Leu*	*Asn*	*Ile*	*Trp*	*Asp*	*Arg*	*Phe*	*Asp*	*Val*	*Phe*	*Cys*	*Thr*	*Leu*	*Gly*	*Ala*	*Thr*	*Thr*	*Gly*	*Tyr*

AAA	GGT	AAT	AGT	XbaI	
AAA	CCA	TTA	TCA	T	AGATC
Lys	*Gly*	*Asn*	*Ser*		

FIG. 5

Linker for combining A8 and VD IV peptides in the same CTA2 fusion

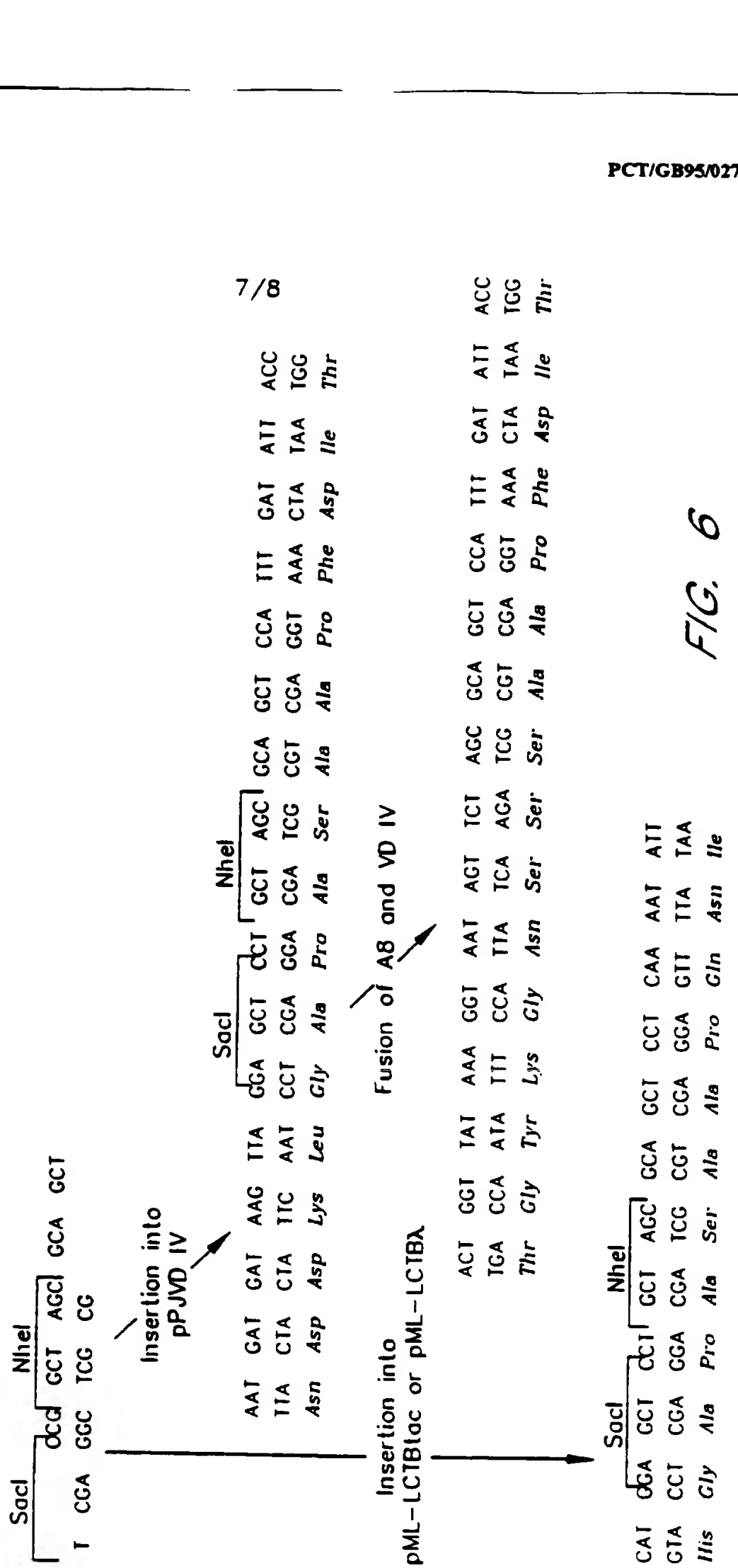

FIG. 6

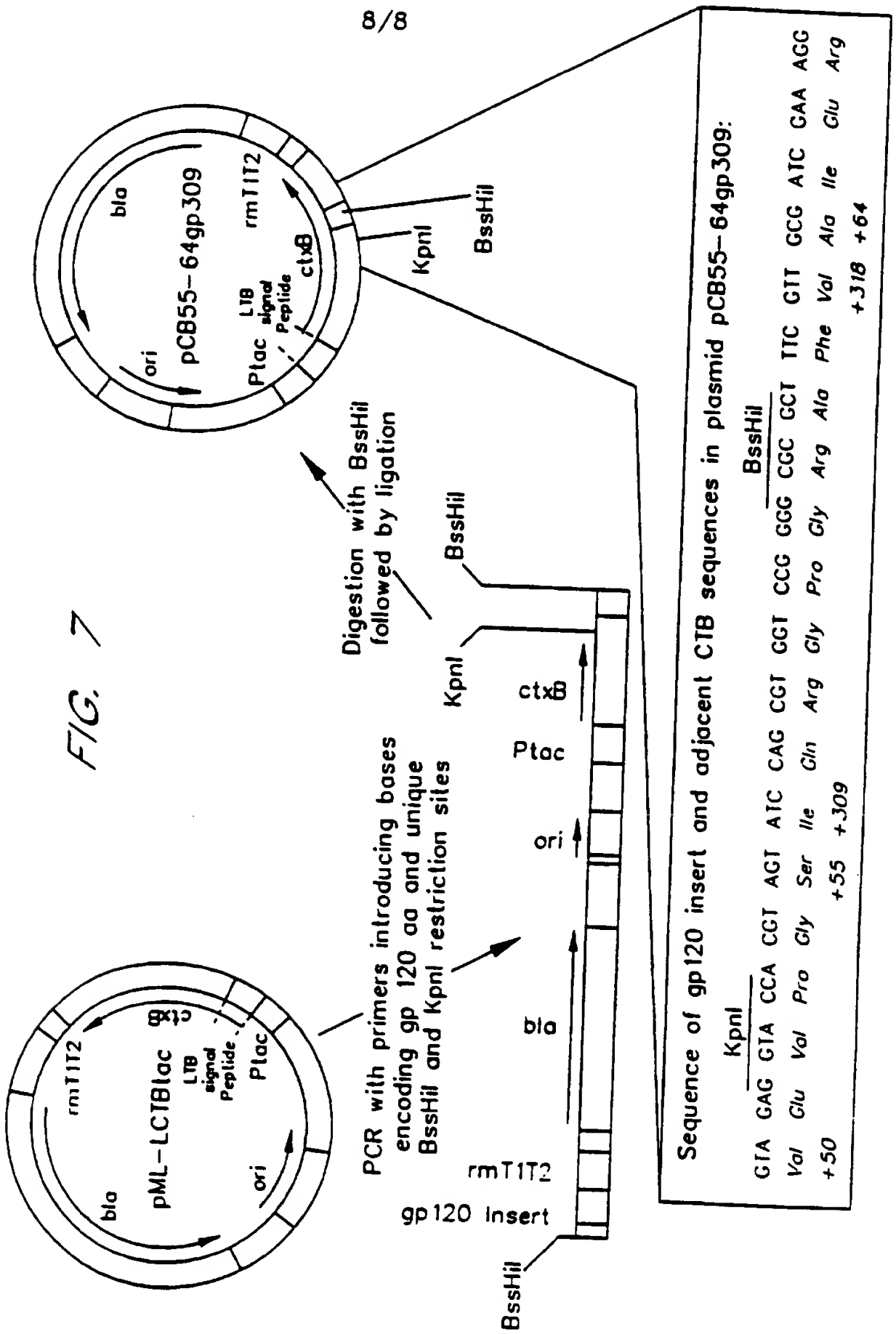
pCB55-64gp309
bla
ori
Ptac
LTB signal Peptide
ctxB
rmT1T2
KpnI
BssHII
Digestion with BssHII followed by ligation
PCR with primers introducing bases encoding gp 120 aa and unique BssHII and KpnI restriction sites
pML-LCTBtac
gp120 Insert
Sequence of gp120 insert and adjacent CTB sequences in plasmid pCB55-64gp309:
Sequence of gp120 insert and adjacent C
GTA GAG GTA CCA CGT AGT ATC CAG CGT
Val Glu Val Pro Gly Ser Ile Gln Arg
+50 +309
CGT CCG GGG CGC GCT TTC GTT GCG ATC GAA AGG
Gly Pro Gly Arg Ala Phe Val Ala Ile Glu Arg
+318 +64

FIG.

INTERNATIONAL SEARCH REPORT

Internat ·l Application No
PCT/uB 95/02708

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C07K14/245 C07K14/28 C07K14/295 C07K14/16
C07K14/02 A61K39/106 A61K39/108 A61K39/118 A61K39/21
A61K39/29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO,A,90 06366 (UNIVERSITY OF LEICESTER) 14 June 1990 see page 1, line 16 - page 2, line 20 see page 3, line 4 - page 4, line 5 see page 6, line 29 - page 7, line 5; examples 1-5 --- -/--	1,4,12, 13,16, 26,33,37 8-10,22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
11 April 1996	07.05.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internat ' Application No
PCT/GB 95/02708

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 07979 (CENTER FOR INNOVATIVE TECHNOLOGY) 13 June 1991 see page 10, last paragraph - page 11, paragraph 1 see page 12, last paragraph - page 15, paragraph 3 see page 18, paragraph 2 - page 19, paragraph 1	1,2, 12-14, 16,26, 27,30, 31,37, 38,43,47

X	EP,A,0 418 626 (TAKEDA CHEMICAL INDUSTRIES, LTD.) 27 March 1991 see column 2, line 38 - column 3, line 12 see column 3, line 26 - column 5, line 26 see column 7, line 41 - line 52; examples 5-11	1,12,13, 16,26,37

X	DATABASE WPI Section Ch, Week 9438 Derwent Publications Ltd., London, GB; Class B04, AN 94-305365 'Fused protein based on endotoxin B sub-unit - and active aminoacid fragment, with Glycine-proline hinge, used to treat virus diseases including HIV, polio, rhinovirus etc.' & JP,A,06 206 900 (WELLCOME FOUND LTD) , 26 July 1994 see abstract	1,2,4, 12-14, 16,17, 37,38

Y	EP,A,0 192 033 (CHIRON CORPORATION) 27 August 1986 see page 3, line 16 - page 4, line 19 see page 5, line 1 - line 17 see page 10, line 30 - page 11, line 22	8-10,22

Y	VACCINE, vol. 11, no. 11, August 1993 GUILDFORD GB, pages 1159-1166, HUA SU ET AL. 'Immunogenicity of a synthetic oligopeptide corresponding to antigenically common T-helper and B-cell neutralizing epitopes of the major outer membrane protein of Chlamydia trachomatis' cited in the application see abstract see page 1165, right column, paragraph 2	8-10,22

2

INTERNATIONAL SEARCH REPORT

...mation on patent family members

Internat...l Application No
PCT/GB 95/02708

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9006366	14-06-90	AU-B-	4754490	26-06-90
		CA-A-	2004738	07-06-90
		EP-A-	0372928	13-06-90
WO-A-9107979	13-06-91	CA-A-	2069106	30-05-91
		EP-A-	0502099	09-09-92
		JP-T-	5503420	10-06-93
EP-A-418626	27-03-91	DE-D-	69005572	10-02-94
		DE-T-	69005572	07-04-94
		JP-A-	3178995	02-08-91
EP-A-192033	27-08-86	NONE		